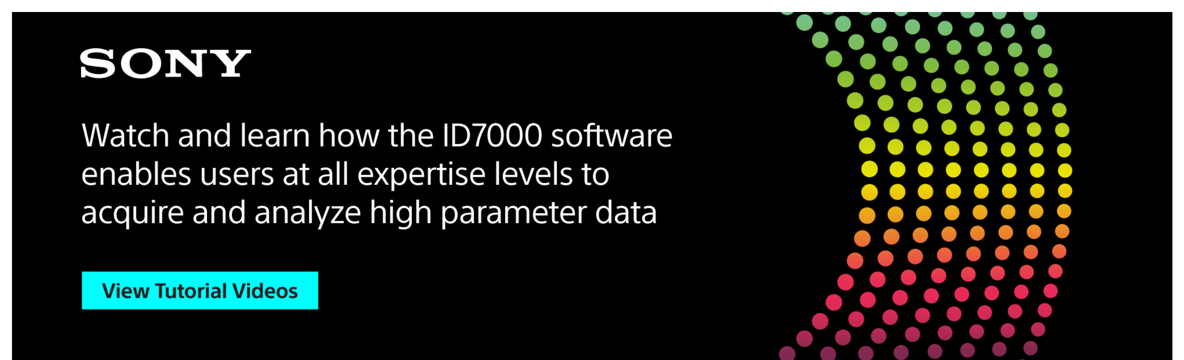

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Vitamin D Receptor and Jak–STAT Signaling Crosstalk Results in Calcitriol-Mediated Increase of Hepatocellular Response to IFN-α

Christian M. Lange,*,† Jérôme Gouttenoire,* François H. T. Duong,‡ Kenichi Morikawa,*,[1] Markus H. Heim,‡ and Darius Moradpour*

Recent clinical research suggests a role for vitamin D in the response to IFN-α–based therapy of chronic hepatitis C. Therefore, we aimed to explore the underlying mechanisms in vitro. Huh-7.5 cells harboring subgenomic hepatitis C virus (HCV) replicons or infected with cell culture–derived HCV were exposed to bioactive 1,25-dihydroxyvitamin D_3 (calcitriol) with or without IFN-α. In these experiments, calcitriol alone had no effect on the HCV life cycle. However, calcitriol enhanced the inhibitory effect of IFN-α on HCV replication. This effect was based on a calcitriol-mediated increase of IFN-α–induced gene expression. Further mechanistic studies revealed a constitutive inhibitory interaction between the inactive vitamin D receptor (VDR) and Stat1, which was released upon stimulation with calcitriol and IFN-α. As a consequence, IFN-α–induced binding of phosphorylated Stat1 to its DNA target sequences was enhanced by calcitriol. Importantly, and in line with these observations, silencing of the VDR resulted in an enhanced hepatocellular response to IFN-α. Our findings identify the VDR as a novel suppressor of IFN-α–induced signaling through the Jak–STAT pathway. ***The Journal of Immunology*, 2014, 192: 6037–6044.**

Chronic infection with the hepatitis C virus (HCV) is highly prevalent in worldwide populations, and a considerable proportion of HCV-infected individuals will develop liver cirrhosis and hepatocellular carcinoma (HCC) within the next decade (1). More extensive screening for HCV infection and improved treatment strategies are necessary to attenuate this expected increase of HCV-related morbidity and mortality. In this regard, the approval of the nonstructural protein 3-4A (NS3-4A) protease inhibitors telaprevir and boceprevir in 2011 was a major breakthrough. Adjunction of these or other directly acting antivirals to pegylated IFN-α and ribavirin results in significantly increased rates of sustained virologic response in HCV genotype 1–infected individuals compared with pegylated IFN-α and ribavirin alone (2). However, owing to a significant risk of drug resistance development, successful triple therapy still depends on the sensitivity to IFN-α of a given patient, as evidenced for example by sustained virologic response rates of only ~30% after telaprevir-based triple therapy in patients with prior null response to pegylated IFN-α and ribavirin (3). More potent triple, quadruple, and all-oral regimens to treat chronic hepatitis C are currently in advanced clinical development (2). Importantly, the individual responsiveness to IFN-α, defined by previous treatment outcome or *IL28B* genotype, remains a determinant of success of several IFN-free, all-oral directly acting antiviral combination therapies as well (3). Hence, modalities to establish an intact endogenous antiviral immune response may remain relevant in the upcoming era of IFN-sparing and IFN-free regimens to treat chronic hepatitis C.

Calcitriol is the bioactive vitamin D metabolite that results from hydroxylation of the precursor cholecalciferol to 25-hydroxyvitamin D_3 (25(OH)D_3) and subsequently to 1,25-dihydroxyvitamin D_3 (calcitriol). By signaling through the vitamin D receptor (VDR), calcitriol serves as an important modulator of innate and adaptive immunity (4, 5). Recent clinical studies have suggested that intact vitamin D signaling may be a determinant of success of pegylated IFN-α and ribavirin therapy in patients with chronic hepatitis C (6–9). Although recent in vitro studies have shown an antiviral effect of distinct vitamin D metabolites against HCV (10, 11), the underlying mechanisms are incompletely understood. Therefore, we investigated a potential inhibitory effect of calcitriol, the bioactive vitamin D metabolite, alone or in combination with IFN-α on HCV RNA replication and infectious particle production in vitro. As a result of these studies, we identified a hitherto unknown link between the VDR and IFN-α–induced signaling through the Jak–STAT pathway. These findings may contribute to the development of novel therapeutic strategies against chronic viral hepatitis and other infectious diseases.

Materials and Methods

Cell culture, subgenomic replicons, cell culture–derived HCV, and plasmids

Huh-7.5 human HCC cells were provided by Charles M. Rice (The Rockefeller University, New York, NY) and cultured in DMEM (Invitrogen)

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The online version of this article contains supplemental material.

Abbreviations used in this article: *CCNC*, cyclin C gene; *CYP24A1*, 1,25-dihydroxyvitamin D_3 24-hydroxylase gene; GAS, IFN-γ–activated sequence; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcc, cell culture–derived hepatitis C virus; IRF9, IFN regulatory factor-9; ISG, IFN-stimulated gene; ISGF3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response element; NS3-4A, nonstructural protein 3-4A; 25(OH)D_3, 25-hydroxyvitamin D_3; siRNA, small interfering RNA; $TCID_{50}$, 50% tissue culture infective dose; VDR, vitamin D receptor.

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containing 10% heat-inactivated FCS. THP-1 cells were cultured in RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated FCS and were differentiated to macrophages in PMA (Sigma-Aldrich) at a concentration of 50 nmol/ml for 72 h. Calcitriol was purchased from Sigma-Aldrich, reconstituted in 100% ethanol, and applied to cells in a final volume corresponding to 0.1% ethanol. Unless stated otherwise, cells in control groups were also cultured in 0.1% ethanol (vehicle). Human IFN-α2a was purchased from Roche. Telaprevir was provided by Johan Neyts (Rega Institute for Medical Research, Leuven, Belgium). Cytotoxicity was assessed by using the WST-1 cell proliferation reagent from Clontech. Subgenomic replicon construct pCon1/SG-Neo(I)/AflII (Con1 strain, genotype 1b) (12) was provided by Charles M. Rice. Subgenomic replicon construct pFK_i389NeoNS3-3′_JFH_dg (JFH1 strain, genotype 2a) (13) and J6/JFH1 (Jc1) full-length construct pFK-JFH1J6C-846_dg (14) were provided by Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany). Cell culture–derived HCV (HCVcc) was produced in Huh-7.5 cells and supernatants titered by 50% tissue culture infective dose ($TCID_{50}$) determination, as described (14, 15). A FLAG-tagged VDR expression construct was constructed as described previously (16) after PCR amplification using VDR-Bsp forward (5′-GATGTCCGGAGAGGCAATGGCGGCC-AGCAC-3′) and VDR-Bam reverse (5′-ATGATGGGATCCGGAGATCT-CATTGCCAAACACT-3′) primers and BspEI-BamHI cloning into a pCMVFLAG-X construct (J.G. and D.M., unpublished), yielding to a pCMVFLAG-VDR construct.

Quantitative real-time PCR

Quantitative real-time PCR was performed with iQ SYBR Green Supermix using a MyiQ iCycler (Bio-Rad), as described previously, including primers for GAPDH mRNA and HCV RNA amplification (17). The following primers were used to quantify mRNA levels of 1,25-dihydroxyvitamin D_3 24-hydroxylase (*CYP24A1*) and cyclin C (*CCNC*) genes: CYP24A1, forward, 5′-GTGGCTCCAGCCAGACCCTA-3′, reverse, 5′-GCGAGGTT-GGTACGAGGTG-3′; CCNC, forward, 5′-ACGGGCTGGGTCTATGG-TCGC-3′, reverse, 5′-GCTCTGCCAAAAGTTC CCTGCCA-3′. Primers for IFN-stimulated gene (ISG) mRNAs (IFI27L, IFI44L, ISG15, OAS, RSAD2) were described previously (18).

Abs

mAb 9E10 against HCV NS5A was provided by Charles M. Rice. Abs against Stat1 p84/p91 (E-23), Stat2 (22), IFN regulatory factor 9 (IRF9; H-143), and VDR (D-6) were from Santa Cruz Biotechnology. Abs against phospho-Stat1 (Tyr^{701}), ISG15 (539442), and phospho-Stat2 (Tyr^{689}) were from Cell Signaling Technology, R&D Systems, and Millipore, respectively. Anti–FLAG M2 and β-actin (AC-15) Abs were from Sigma-Aldrich. Alexa Fluor 488– and 594–conjugated secondary Abs were from Life Technologies.

Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were lysed by three freeze-and-thaw cycles in a buffer containing 0.1% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, and 50 mM Tris·HCl (pH 8.0). Binding and washing were performed in TBS containing 0.05% Tween 20, 1 mM vanadate, and 1× cOmplete protease inhibitor cocktail (Roche). Immunoprecipitation was performed using protein G Dynabeads (Invitrogen). Immunoblotting was performed as described (17).

Immunofluorescence

Immunofluorescence staining was performed as described (19), and images were acquired on a Leica SP5 confocal laser scanning microscope.

EMSA

EMSA was performed as described (20).

Gene silencing

Gene silencing in Huh-7.5 cells was performed in 12-well plates using predesigned small interfering RNAs (siRNAs) from Applied Biosystems. Single siRNAs (s1477 or s14779 for VDR, s277 or s278 for Stat1, s13529 or s13530 for Stat2, or nontargeting control siRNA) were transfected at a final concentration of 5 nM using Lipofectamine RNAiMAX (Invitrogen).

Results

Calcitriol alone has no direct antiviral activity against HCV RNA replication and infectious particle production in vitro

Huh-7.5 human HCC cells harboring subgenomic HCV replicons or infected with HCVcc were exposed to increasing concentrations (0.1–100 nM) of calcitriol for up to 72 h. The lowest concentration (0.1 nM) was chosen because it represents the serum concentration that is considered to be optimal in humans (21). We first excluded relevant effects of calcitriol on cell viability throughout the dose range tested and confirmed intact VDR signaling in Huh-7.5 cells by quantifying the expression of vitamin D target genes *CYP24A1* and *CCNC,* which were induced and repressed by calcitriol, as expected (Fig. 1A, 1B) (22). However, no relevant effect of calcitriol on HCV RNA replication was observed by quantitative real-time PCR for intracellular viral RNA and immunoblot for viral nonstructural protein 5A (NS5A) in Huh-7.5 cells harboring subgenomic HCV replicons (Fig. 1C). To exclude a direct effect of calcitriol on the early or late steps of the viral life cycle, that is, entry and infectious particle assembly as well as release, Huh-7.5 cells were exposed for 48 h to 0.1–100 nM calcitriol, followed by HCVcc infection during continued calcitriol exposure. Forty-eight hours after infection, cell culture supernatants were collected and titered by $TCID_{50}$ determination. As shown in Fig. 1D, calcitriol did not significantly affect infectious particle production or NS5A expression, whereas telaprevir as a control strongly inhibited HCV RNA replication and virus production. Taken together, these experiments did not reveal any relevant direct antiviral effect of calcitriol at doses within and exceeding the physiological range in vitro, under conditions where cell viability is not affected and vitamin D is active.

Calcitriol enhances the inhibitory effect of IFN-α on HCV RNA replication in vitro

Because we did not observe any direct antiviral effect of calcitriol against HCV, we tested whether calcitriol may enhance IFN-α–induced suppression of HCV replication. In Huh-7.5 cells containing either HCV genotype 1b or 2a replicons, treatment with calcitriol in combination with IFN-α resulted in a significantly more pronounced suppression of HCV RNA replication as compared with IFN-α alone (Fig. 1E). This was especially evident when relatively low doses of IFN-α were applied.

Calcitriol enhances IFN-α–induced ISG expression

To explore the mechanism of the enhanced antiviral effect of IFN-α in the presence of calcitriol, we first determined the expression level of a number of ISGs in naive Huh-7.5 cells after treatment with calcitriol and/or IFN-α. A consistent increase in the expression level of ISGs was observed after combination treatment with calcitriol plus IFN-α as compared with IFN-α alone (Fig. 2). In contrast, calcitriol alone had only a weak effect on the induction of ISGs (Fig. 2). The enhanced induction of ISGs in the presence of calcitrol was observed at early time points (4 and 8 h of treatment) and appeared to be long-lasting after treatment with IFN-α (24 h). Importantly, low doses of calcitriol (0.1 nM) were sufficient to increase the response to IFN-α (Fig. 2). These results were similar in Huh-7.5 cells containing HCV genotype 1b replicons (Supplemental Fig. 1). Furthermore, calcitriol-mediated enhancement of IFN-α–induced ISG expression was also evident at the protein level, as assessed exemplarily by immunoblot analysis of ISG15 expression (Fig. 3).

In line with the above observations, DNA binding of the IFN-α–induced ISG factor 3 (ISGF3) transcription factor complex to its DNA target sequence (i.e., IFN-stimulated response element [ISRE]), assessed by EMSA, was more pronounced in the presence of calcitriol as compared with IFN-α alone (Fig. 4A). Additionally, calcitriol led to a moderate increase of IFN-α–induced homo- and heterodimers of Stat1 and Stat3, which bind to IFN-γ–activated sequence (GAS) response elements (Fig. 4B).

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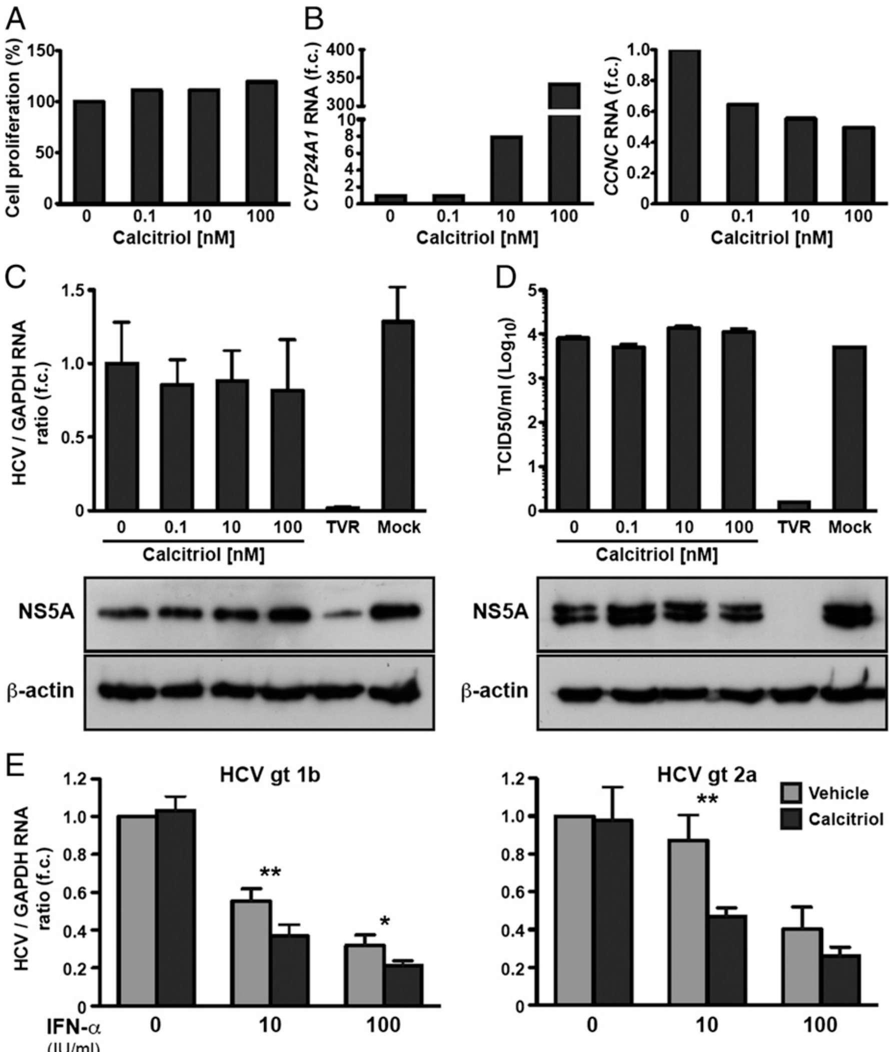

FIGURE 1. Calcitriol alone has no direct antiviral activity in vitro but enhances the antiviral effect of IFN-α on HCV replication. (**A**) Viability of Huh-7.5 cells was not affected by exposure for 48 h to increasing concentrations of calcitriol, as assessed by the WST-1 cell proliferation reagent. (**B**) Quantification of mRNA levels of calcitriol target genes *CYP24A1* (upregulated, *left panel*) and *CCNC* (downregulated, *right panel*) after exposure to calcitriol for 48 h confirms functional vitamin D receptor signaling in Huh-7.5 cells. f.c., fold change. (**C**) HCV RNA levels relative to GAPDH mRNA in Huh-7.5 cells harboring a subgenomic JFH1 replicon after exposure to calcitriol at the indicated concentrations for 48 h. Immunoblots for HCV NS5A and β-actin performed on the same cell pellets are shown at the bottom. Results were comparable after 24 and 72 h as well as in Huh-7.5 cells harboring a subgenomic Con1 replicon. (**D**) Huh-7.5 cells were cultured 48 h in the presence of 0–100 nM calcitriol, as indicated, followed by Jc1 HCVcc infection at a moiety of infection of 1 under continued calcitriol exposure. $TCID_{50}$/ml in supernatants was determined 48 h after infection. Mock indicates no supplement to cell culture, whereas 0 nM calcitriol indicates supplementation with 0.1% ethanol as carrier. TVR, telaprevir (2.5 μM). (**E**) Huh-7.5 cells harboring subgenomic HCV genotype 1b (Con1) or genotype 2a (JFH1) replicons were cultured for 6 h in the presence of 0.1 nM calcitriol (dark gray columns) or 0.1% ethanol as carrier (light gray columns), followed by stimulation for 24 h with IFN-α at the indicated concentrations with or without 0.1 nM calcitriol. HCV RNA levels normalized to GAPDH mRNA are expressed relative to untreated cells. Standard deviations of two experiments performed in triplicate are shown. $^{*}p < 0.1$, $^{**}p < 0.05$.

To test whether the enhancing effect of calcitriol on IFN-α signaling is restricted to hepatocytes, we assessed ISG induction in differentiated macrophages derived from the monocytic leukemia cell line THP-1, which are known to express relevant levels of VDR (4). As shown in Fig. 5, calcitriol had a comparable effect on IFN-α–induced ISG expression in THP-1 cells as was observed in Huh-7.5 cells.

Calcitriol does not increase IFN-α–induced phosphorylation of Stat1 or Stat2

To further investigate the underlying mechanism of the calcitriol-mediated increase of IFN-α–induced ISG induction, we first quantified protein levels of components of ISGF3, a heterotrimeric complex of phospho-Stat1, phospho-Stat2, and IRF9, which serves as transcription factor of type I IFN–induced ISGs. Treatment of Huh-7.5 cells with 100 IU/ml IFN-α for 30 min resulted in substantial phosphorylation of Stat1 and Stat2 (Fig. 6). However, addition of calcitriol did not increase Stat1 or Stat2 phosphorylation, and no increased expression of total Stat1, total Stat2, or IRF9 was observed after calcitriol treatment (Fig. 6).

VDR constitutively interacts with Stat1

The observations reported above suggest a link between calcitriol and IFN-α beyond Stat1/Stat2 phosphorylation. Therefore, we performed immunoprecipitation experiments to test for complex formation between VDR and Stat1. Using protein lysates from naive Huh-7.5 cells, we observed a constitutive association between the VDR and Stat1 in the absence of calcitriol and IFN-α

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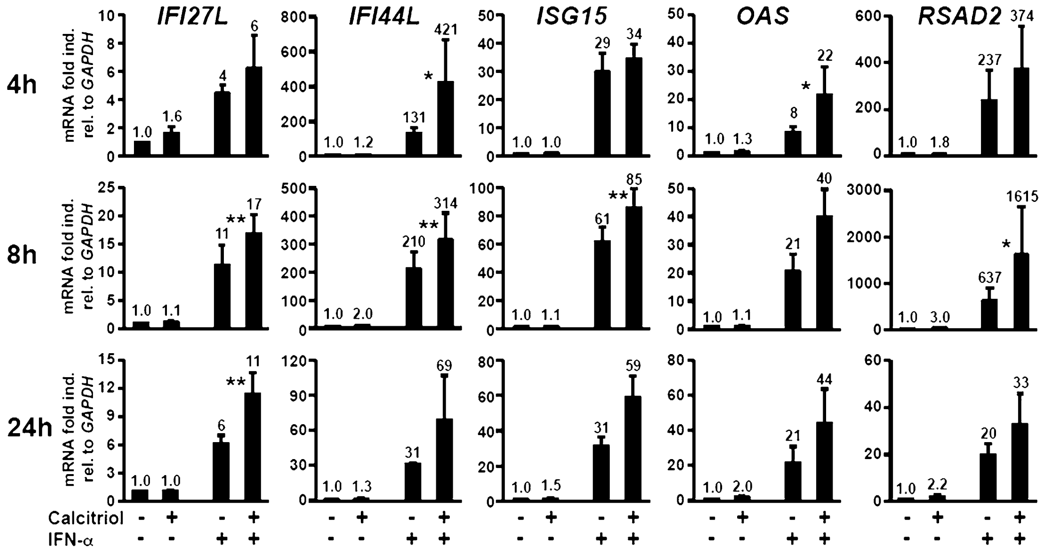

FIGURE 2. Calcitriol enhances hepatocellular ISG induction. Huh-7.5 cells were cultured for 6 h in the absence or presence of 0.1 nM calcitriol, followed by incubation without or with 100 IU/ml IFN-α with or without 0.1 nM calcitriol for 4 h (*upper panel*), 8 h (*middle panel*), or 24 h (*lower panel*). mRNA expression of the indicated ISGs was normalized to GAPDH mRNA and expressed as fold induction relative to untreated cells. Standard errors of the mean of six independent experiments are shown. *$p < 0.1$, **$p < 0.05$.

(Fig. 7). Interestingly, the association between VDR and Stat1 decreased after IFN-α treatment alone and almost completely disappeared upon IFN-α treatment in combination with calcitriol.

Consistent with the results from the EMSA, immunoblot, and immunoprecipitation analyses above, increased nuclear phospho-Stat1 was observed in cells treated with IFN-α and calcitriol as compared with IFN-α alone (Fig. 8). Additionally, heterologous overexpression of the VDR reduced the nuclear localization of phospho-Stat1, presumably by retaining phospho-Stat1 in the cytosol (Fig. 8). Because this molecular mechanism would be independent from de novo protein synthesis, we tested whether simultaneous administration of calcitriol together with IFN-α (instead of preincubation with calcitriol for 6 h, applied thus far) is also sufficient to enhance IFN-α–induced ISG expression. Indeed, this treatment regimen did not reduce to effect of calcitriol on IFN-α signaling (Fig. 9A). Furthermore, the effect of calcitriol on IFN-α–induced ISG expression was retained when Huh-7.5 cells were treated with cycloheximide, an inhibitor of protein synthesis (Fig. 9B).

Silencing of VDR gene expression results in increased responsiveness to IFN-α

The constitutive interaction between VDR and Stat1, which was found to dissociate after stimulation with IFN-α and calcitriol, may point to an inhibitory effect of the inactive VDR on Jak–STAT signaling. To test this hypothesis, we silenced *VDR* gene expression by transfecting siRNAs into Huh-7.5 cells. Silencing of *VDR* gene expression was achieved 4 d after transfection and confirmed by quantification of VDR mRNA (data not shown) and protein level (Fig. 10A, *lower panel*). Subsequently, Huh-7.5 cells were treated with IFN-α with or without calcitriol, and mRNA of two selected ISGs was quantified after a 4-h treatment period. Silencing of *Stat1* and *Stat2* gene expression was performed accordingly as a control. Stimulation of VDR siRNA-treated cells with IFN-α resulted in significantly stronger induction of IFI27L and IFI44L mRNA expression, compared with cells treated with control siRNA (Fig. 10A). In contrast, siRNAs targeting Stat1 or Stat2 mRNA significantly impaired the response to IFN-α (Fig. 10A). In line with these results, immunofluorescence analyses showed that silencing of VDR gene expression significantly increased nuclear accumulation of pStat1 after stimulation with IFN-α (Fig. 10B). Taken together, these data support the hypothesis of an

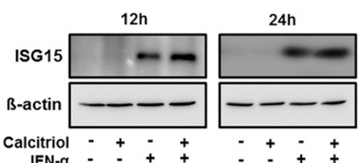

FIGURE 3. Calcitriol increases ISG15 protein levels upon stimulation with IFN-α. Huh-7.5 cells were exposed to 250 IU/ml IFN-α after a pretreatment period of 6 h with calcitriol (0.1 nM). Immunoblots for assessing the protein levels of ISG15 and β-actin were performed after incubation with IFN-α for 12 or 24 h.

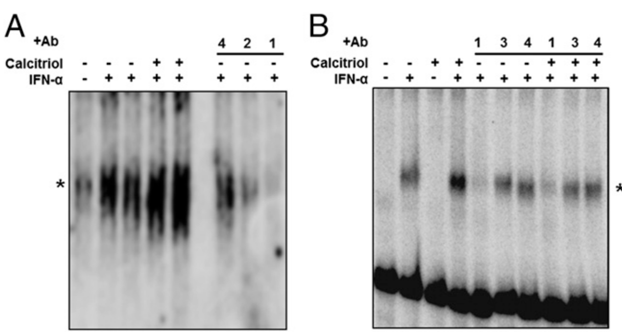

FIGURE 4. Calcitriol enhances DNA binding of ISGF3 and phosphorylated Stat1 upon stimulation with IFN-α. Huh-7.5 cells were incubated without or with 1000 IU/ml IFN-α for 30 min after a pretreatment period of 6 h with 1 nM calcitriol or vehicle (0.1% ethanol). Nuclear extracts were analyzed by EMSA using an ISRE oligonucleotide probe (**A**) or the GAS-element containing SIE-m67 oligonucleotide probe (**B**). Increased binding of Stat1/Stat2 heterodimers (*) upon stimulation with IFN-α was observed in the presence of calcitriol, compared with IFN-α alone. Antisera specific to 1) Stat1, 2) Stat2, 3) Stat3, or 4) VDR were used to shift Stat dimers. No shift of Stat dimers was observed in the presence of antiserum specific to VDR. In contrast, the bands indicating binding of Stat dimers dissapeared in the presence of antiserum specific to Stat1 or Stat2. Hence, VDR does not seem to be involved directly in DNA binding of Stat dimers to ISRE and GAS elements.

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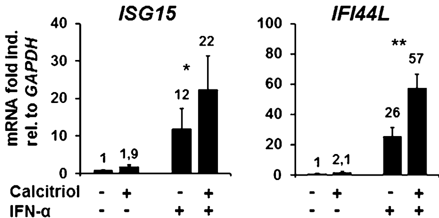

FIGURE 5. Calcitriol enhances ISG induction in THP-1 cells. THP-1 cells were differentiated to macrophages in PMA at a concentration of 50 nmol/ml for 72 h and then cultured for 6 h in the absence or presence of 0.1 nM calcitriol, followed by incubation without or with 100 IU/ml IFN-α with or without 0.1 nM calcitriol for 6 h. mRNA expression of the indicated ISGs was normalized to GAPDH mRNA and expressed as fold induction relative to untreated cells. Standard errors of the mean of six independent experiments are shown. $^{*}p < 0.1$, $^{**}p < 0.05$.

inhibitory role of VDR in IFN-α–induced signaling through the Jak–STAT pathway.

Discussion

Type I IFNs are key players in the innate immune response against numerous pathogens, including HCV (23). Type I IFN signaling through the heterodimeric type I IFN receptor results in Jak-dependent phosphorylation of Stat1 and Stat2, which subsequently heterodimerize to form the ISGF3 complex in association with IRF9 (23, 24). ISGF3 serves as a major transcription factor to induce a variety of ISGs, which together orchestrate an antiviral cellular state (25). However, ISGF3-induced ISG expression can be modulated, as it has been shown, for example, that p38 activity can enhance ISGF3-mediated gene expression (26). In the present study, we identify calcitriol and the calcitriol receptor VDR as modulators of IFN-α–induced Jak–STAT signaling.

Calcitriol is a steroid hormone that exerts its pleiotropic biological effects mainly via activation of the VDR, a member of the nuclear receptor family, which regulates the transcription of numerous genes (21, 22). In the present study, we identify the VDR as a novel suppressor of IFN-α–induced signaling through the Jak–STAT pathway. VDR-mediated suppression of Jak–STAT signaling involves a constitutive interaction between the VDR and Stat1 in unstimulated Huh-7.5 cells, which is released after stimulation with calcitriol and IFN-α. As a consequence, calcitriol enhances the effects of IFN-α on the expression of ISGs as well as on HCV replication. These effects were not mediated by increased phosphorylation of Stat1 or Stat2 in the presence of calcitriol, but rather by increased nuclear trafficking/DNA binding of phospho-Stat1. Furthermore, a substantially increased hepatocellular response to IFN-α after silencing of VDR gene expression confirmed the inhibitory role of the inactive VDR in IFN-α–induced Jak–STAT signaling. The divergent net effect of calcitriol and the inactive VDR on IFN-α–induced Jak–STAT signaling sheds light on a novel mode of receptor crosstalk between two different signaling pathways, in which a member of the nuclear receptor family plays a key role in its nonstimulated state. In this regard, it may be important that VDR is expressed not in all tissues and that VDR expression in some cell types (e.g., in macrophages) is, at least partially, inducible (4, 22). Hence, the crosstalk between VDR and IFN-α signaling observed in the present study may well constitute a dynamic and tissue- or cell-specific way to control IFN-α signaling. Additional research is needed to further explore these interesting questions.

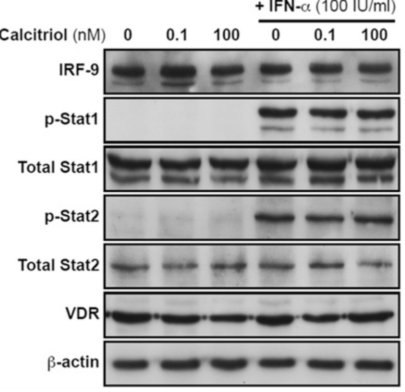

FIGURE 6. Calcitriol does not enhance phosphorylation of Stat1 or Stat2. Huh-7.5 cells were exposed to 100 IU/ml IFN-α for 30 min after a pretreatment period of 6 h with calcitriol at the indicated concentrations. Immunoblots for the different components of the ISGF3 complex as well as for the VDR and β-actin are shown.

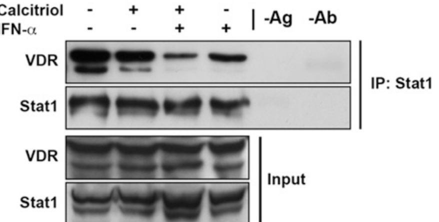

FIGURE 7. Stat1 constitutively interacts with the vitamin D receptor. Huh-7.5 cells were cultured in the presence or absence of 1 nM calcitriol, followed by incubation without or with 1000 IU/ml IFN-α for 30 min. Immunoprecipitation (IP) was performed using a rabbit polyclonal Ab against total Stat1, and precipitates were immunoblotted using a mouse mAb against the VDR or against total Stat1 (*upper panel*). Samples containing either no Ag or no Ab during the IP were included as controls. Immunoblots for Stat1 and VDR from cell lysates subjected to IP are shown at the bottom.

Direct molecular or indirect regulatory interactions between members of the nuclear receptor family and Jak–STAT signaling pathways have been observed previously. For example, a synergistic effect of glucocorticoids on Stat5-mediated gene expression has been demonstrated, which depended on molecular interactions between Stat5 and the glucocorticoid receptor (27). However, in contrast to our observations, Stat5 and the glucocorticoid receptor formed a molecular complex after stimulation of both receptors with prolactin and dexamethasone, respectively, and not in the unstimulated state. Another study in osteoblast cell lines has revealed a synergism between calcitriol and Stat5-mediated growth hormone signaling, which may involve complex effects of calcitriol on Stat5-induced suppressor of cytokine signaling 3 expression as well as on nuclear export of activated Stat5 (28). Finally, a previous study reported a crosstalk between VDR and Stat1 signaling pathways, which affected calcitriol-induced expression of VDR target genes in macrophages (29). Together with our observations, these studies suggest an important role for a crosstalk between Jak–STAT– and nuclear receptor–mediated signaling pathways in various conditions.

We have observed a substantially increased response to IFN-α after silencing of *VDR* gene expression. Compared to the strong effect of silencing of *VDR* gene expression on ISG induction, the contribution of calcitriol to ISG induction and suppression of HCV replication was relatively moderate. Therefore, the inactive VDR might be considered as the predominant regulatory element in this context, whereas activation of VDR with calcitriol may allow for fine tuning. In view of these findings, it may be relevant to test whether other drugs (e.g., nonhypercalcemic VDR agonists

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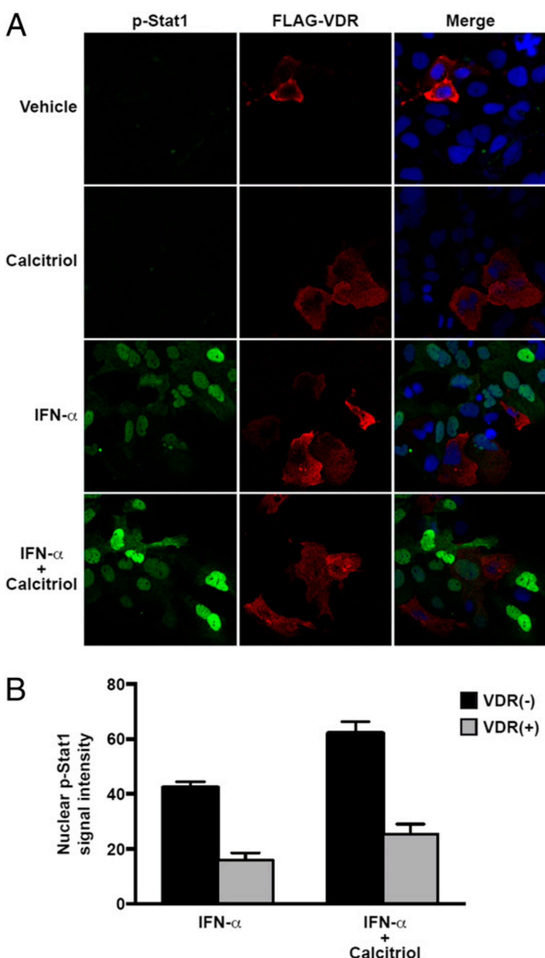

FIGURE 8. IFN-α–induced nuclear localization of phospho-Stat1 is enhanced by calcitriol and repressed by VDR overexpression. (**A**) Huh-7.5 cells were transfected with a FLAG-tagged VDR expression construct, followed by incubation with 0.1 nM calcitriol or vehicle (0.1% ethanol) for 6 h and stimulation with 1000 IU/ml IFN-α for 30 min. Cells were fixed with methanol, followed by standard indirect immunofluorescence analysis using rabbit mAb 58D6 against phospho-Stat1 (Cell Signaling Technology) and mouse mAb anti-FLAG M2 (Sigma-Aldrich) as primary Abs as well as Alexa Fluor 488– and 594–conjugated secondary Abs, respectively (Life Technologies). Nuclei were stained with DAPI (AppliChem). Original magnification ×40. (**B**) Nuclear phospho-Stat1 fluorescence intensity was quantified in each cell of five randomly selected fields comprising ~50 cells each by using ImageJ software. Results were analyzed separately for cells overexpressing FLAG-VDR (VDR(+)) or not (VDR(−)) using GraphPad Prism software. Standard errors of the mean are shown. Values of p are <0.01 for the following comparisons: IFN-α, VDR(+) versus IFN-α, VDR(−); IFN-α plus calcitriol, VDR(+) versus IFN-α plus calcitriol, VDR(−); IFN-α, VDR(+) versus IFN-α plus calcitriol, VDR(+); IFN-α, VDR(−) versus IFN-α plus calcitriol, VDR(−).

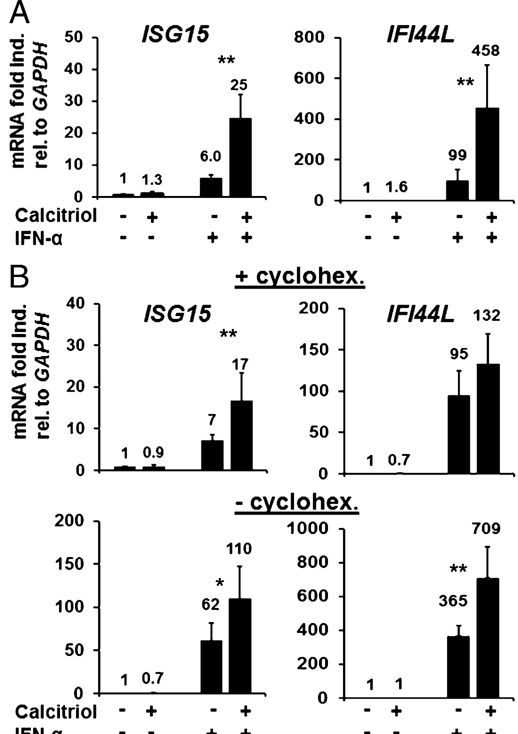

FIGURE 9. Calcitriol enhances IFN-α–stimulated ISG induction independently from de novo protein synthesis. (**A**) Calcitriol enhances ISG induction during simultaneous treatment with IFN-α. Huh-7.5 cells were stimulated simultaneously with placebo, 0.1 nM calcitriol, or 100 IU/ml IFN-α, as indicated. After 6 h, mRNA expression of the indicated ISGs was normalized to GAPDH mRNA and expressed as fold induction relative to untreated cells. (**B**) Calcitriol enhances ISG induction in the presence of cycloheximide, an inhibitor of de novo protein synthesis. Huh-7.5 cells were preincubated with (*upper panel*) or without (*lower panel*) cycloheximide (50 μg/ml) for 12 h and then cultured for 6 h in the absence or presence of 0.1 nM calcitriol, followed by incubation without or with 100 IU/ml IFN-α with or without 0.1 nM calcitriol for 6 h. mRNA expression of the indicated ISGs was normalized to GAPDH mRNA and expressed as fold induction relative to untreated cells. Standard errors of the mean of six independent experiments are shown. $*p < 0.1$, $**p < 0.05$.

such as paricalcitol) may target the VDR–Stat1 interaction more efficiently than calcitriol itself. Indeed, in preliminary experiments we have observed a synergistic effect between synthetically developed VDR agonists and IFN-α on ISG expression (C.M.L., unpublished data). Such investigations might lead to novel therapeutic strategies to overcome IFN resistance in perhaps various settings.

The in vivo relevance of our findings is currently unknown. A recent placebo-controlled proof-of-principle clinical study demonstrated that the experimentally well-defined effect of calcitriol to enhance the TLR-mediated ability of macrophages to combat *Mycobacterium tuberculosis* can indeed translate into a favorable treatment outcome of lung tuberculosis due to vitamin D supplementation (4, 30). Retrospective analyses of HCV-infected patients as well as a small, non-placebo–controlled clinical trial suggested a possible benefit of intact vitamin D signaling in the response of IFN-α–based therapy of chronic hepatitis C, although it is still under debate whether serum levels of the calcitriol precursor 25(OH)D_3 are a predictor of treatment outcome (6–9). The findings of our present study support further evaluation of vitamin D supplementation (or, in theory, specific targeting of the VDR–Stat1 interaction) before/during antiviral therapy of HCV infection. Importantly, an improvement of effects of endogenous or exogenous IFN-α still appears to be relevant in the upcoming era of IFN-sparing and IFN-free treatment regimens (3). In this regard, it appears to be crucial that we observed an increased hepatocellular response to IFN-α even in the presence of low concentrations of calcitriol, which can be realistically achieved under optimal conditions in human serum (21). Furthermore, the observed enhancing effect of calcitriol on IFN-α signaling in differentiated macrophages derived from the monocytic leukemia cell line THP-1 may indicated that the crosstalk between VDR and IFN-α signaling may impact indirectly on HCV as well via modulating macrophage-mediated antiviral immunity. Further

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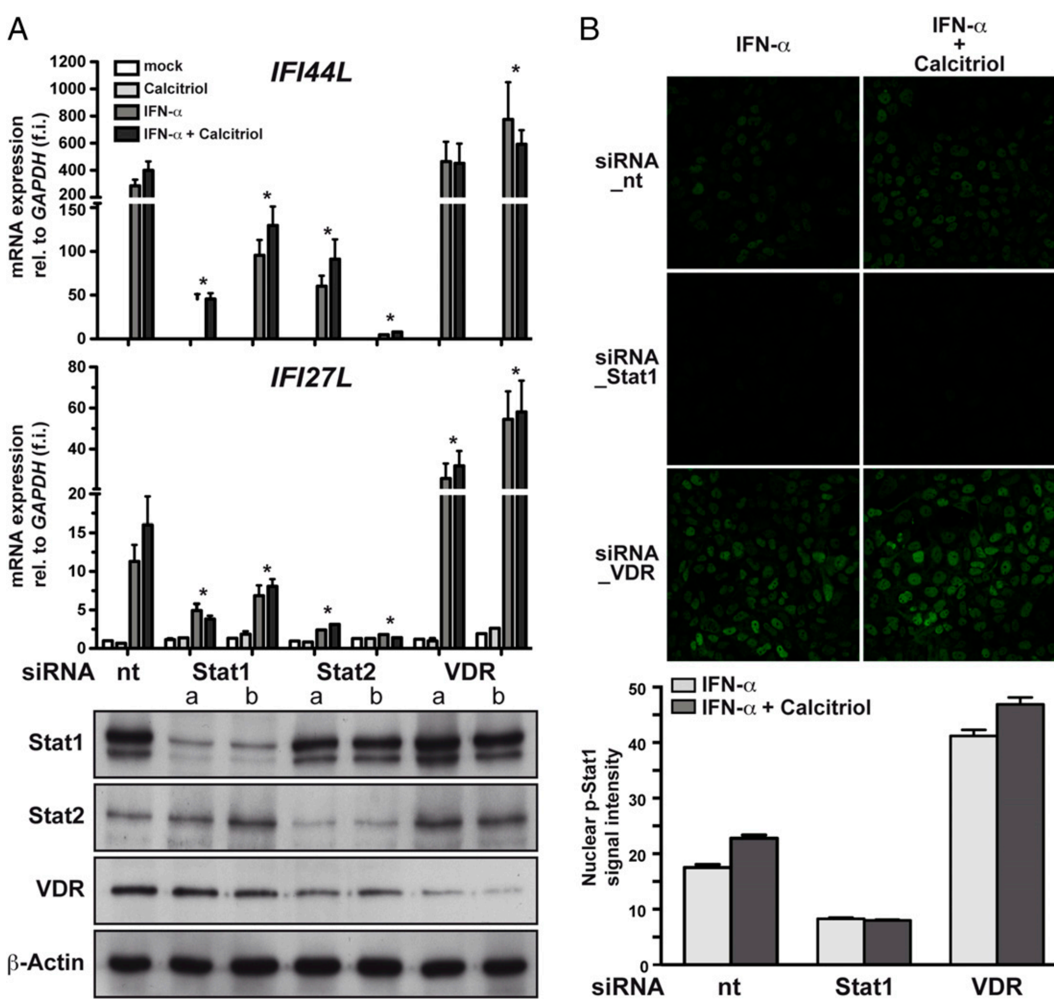

FIGURE 10. Silencing of *VDR* gene expression results in increased hepatocellular response to IFN-α. (**A**) Huh-7.5 cells were transfected with either nontargeting siRNA (nt) or two different siRNAs each (a or b, as specified in *Materials and Methods*) targeting Stat1, Stat2, or VDR. Ninety-six hours later, cells were treated with or without calcitriol (0.1 nM) for 6 h, followed by incubation for 4 h with or without 100 IU/ml IFN-α. mRNA expression of the indicated ISGs was normalized to GAPDH mRNA and expressed as fold induction (f.i.) relative to untreated cells that were transfected with nontargeting siRNA (*upper panels*). SDs of two independent experiments, each performed in triplicate, are shown. $*p < 0.05$ for comparison with nt siRNA-transfected cells. Statistics for comparisons between cells treated with IFN-α plus vehicle versus IFN-α plus calcitriol are as follows: *IFI44L*, $p = 0.11$ for nt, $p > 0.2$ for VDRa and VDRb; *IFI27L*, $p = 0.03$ for nt, $p > 0.2$ for VDRa and VDRb. Silencing of Stat1, Stat2, and VDR expression was confirmed 96 h after siRNA transfection (*bottom panel*). (**B**) Huh-7.5 cells were transfected with either nontargeting siRNA (nt) or one siRNA targeting Stat1 or VDR, followed by incubation with 0.1 nM calcitriol or vehicle (0.1% ethanol) for 6 h and stimulation with 1000 IU/ml IFN-α for 30 min. Cells were fixed with methanol, followed by standard indirect immunofluorescence analysis using rabbit mAb 58D6 against phospho-Stat1 (Cell Signaling Technology) and with Alexa Fluor 488–conjugated secondary Abs (Life Technologies). Nuclei were stained with DAPI (AppliChem). Images were acquired in strictly similar acquisition settings on a Leica SP5 confocal laser scanning microscope. Nuclear phospho-Stat1 fluorescence intensity was quantified in each cell of ~200 cells by using ImageJ software. Results were analyzed separately for cells transfected with siRNA nt, VDR, and Stat1 and treated or not by calcitriol using the GraphPad Prism software. Standard errors of the mean are shown. A p value of <0.01 applies for the following comparisons: siRNA_nt IFN-α versus siRNA_nt IFN-α plus calcitriol; siRNA_VDR IFN-α versus siRNA_VDR IFN-α plus calcitriol; siRNA_nt IFN-α versus siRNA_VDR IFN-α; siRNA_nt IFN-α plus calcitriol versus siRNA_VDR IFN-α plus calcitriol.

studies are warranted to address the implications of these initial observations.

Importantly, we did not observe any direct antiviral effect of calcitriol alone in numerous experiments. This finding is in line with a previous study by Matsumura et al. (11), but in contrast to a study by Gal-Tanamy et al. (10), which has reported an antiviral effect of supraphysiologic doses of calcitriol alone in vitro. The discrepancies between these studies remain unclear, but they may be explained by a different HCV construct (HJ-3-5) used by Gal-Tanamy et al. (10), which might be more sensitive to the very moderate induction of ISGs by calcitriol alone. However, it appears important that both Gal-Tanamy et al. and Matsumura et al. have observed a significant direct antiviral effect of the calcitriol precursor $25(OH)D_3$. Direct inhibition of HCV by $25(OH)D_3$ was further supported by the occurrence of a resistance mutation in the HCV NS3-4A protease during continuous exposure to $25(OH)D_3$ in the study by Matsumura et al. (11). Pleiotropic effects of vitamin D and its analogs are well known (22). Altogether, the results of our present study as well as those of the previous studies by Gal-Tanamy et al. and Matsumura et al. indicate that the calcitriol precursor $25(OH)D_3$, which has no relevant affinity to VDR, suppresses HCV directly (perhaps by targeting NS3-4A), whereas the VDR agonist calcitriol impacts on HCV via modulating cellular responsiveness to endogenous and exogenous type I IFN.

In conclusion, our findings reveal a hitherto unknown link between vitamin D metabolism and IFN-α–induced signaling through the Jak–STAT pathway, in which an association between nonstimulated VDR and Stat1 appears to play a key role. These

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findings may contribute to the development of novel therapeutic strategies against chronic hepatitis C and possibly also chronic hepatitis B and D as well as other infectious diseases in which innate immune responses play an important role.

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Disclosures

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