Janus kinase-inhibition modulates hepatitis E virus infection

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self-limiting in most of the cases, immunocompromised individuals are at high risk to develop chronic hepatitis resulting in liver fibrosis, cirrhosis and fulminant liver failure (Dalton et al., 2009). To date, anti-HEV therapy is limited to administration of pegylated interferon-α (pegIFN-α) and off-label use of ribavirin (2018; Kinast et al., 2019). The administration and clinical effect of pegIFN-α on HEV replication implies that the innate immune system plays a major role in the course of an HEV infection (Kamar et al., 2010). IFNs trigger the JAK-STAT (Janus kinase - signal transducer and activator of transcription) signaling pathway, resulting in activation of a cellular immune response and the expression of a panoply of interferon-stimulated genes (ISGs) (Schneider et al., 2014). Inhibitors of JAK can block those pathways, resulting in a certain degree of immune-modulation/suppression. Importantly, a novel class of selective JAK inhibitors (JAKis) has been introduced recently for the treatment of autoimmune inflammatory rheumatic diseases (AIRD) (Smolen et al., 2020). AIRD are chronic diseases characterized by the production of autoantibodies against post-translationally modified proteins and infiltration of the synovial membrane in multiple joints with T cells, B cells, and monocytes (Aletha and Smolen, 2018). Clinical features are synovial inflammation and hyperplasia but also systemic complications such as pulmonary or cardiovascular disorders can arise (McInnes and Schett, 2011). Of note, JAK1 treatment in AIRD patients has been associated with reactivations of herpesviruses, such as varicella zoster virus and human cytomegalovirus pointing to a relationship between JAKi-mediated immune modulation and viral infection/reactivation (Smolen et al., 2020). Reactivation was also evaluated and discussed for hepatitis B virus (HBV), a significant health burden and hepatotropic pathogen. (Harigai et al., 2020; Winthrop et al., 2020). Recently, association of an acute HEV infection in an AIRD patient treated with the JAK1 inhibitor baricitinib was reported, further raising the question whether JAKis can affect the fate of HEV infections (Valor-Méndez et al., 2021). Given that JAKi therapy is comparatively new in the treatment of AIRD, clinical and molecular evaluation of the role of JAKis during HEV infection is limited. To address this sparse zone in translational HEV research, we assessed HEV infection in AIRD patients treated with JAKis, evaluated the consequence of JAKi therapy on ex vivo HEV infection in primary human hepatocytes (PHHs) and dissected the transcriptional response of those cells.

2. Materials and methods

All material and methods can be found in the supplementary section.

3. Results

3.1. An AIRD patient under baricitinib therapy with hepatitis E

During our study, a 53-year-old female patient with rheumatoid arthritis (RA) presented to her rheumatologist for diagnostic evaluation of elevated liver enzymes that had been diagnosed on routine blood testing (Fig. 1). Aspartate transaminase (AST) was 300 (norm <35) U/L, alanine transaminase (ALT) 278 (norm <35) U/L and Gamma-glutamyltransferase (GGT) 445 (norm <40) U/L. Bilirubin and cholinesterase values were not elevated. C-reactive protein (CRP) value and erythrocyte sedimentation rate (ESR) were elevated as a sign of active inflammation. Red blood cell count (RBC) and lymphocyte numbers were within the normal range. In a preceding test, four weeks prior to the presentation, GGT was slightly elevated at 58 U/L, AST and ALT were within the normal range. Rheumatoid arthritis had been diagnosed 29 years before and had been treated with chloroquine, D-penicillamine, sulfasalazine, methotrexate, etanercept and steroids in the past. The antirheumatic therapy had been changed to baricitinib due to disease activity four months before. Upon presentation, the patient did not report of any symptoms. The inflammatory activity of the rheumatoid arthritis was suppressed. Diagnostic workup revealed anti-HEV IgM an IgG antibodies, HEV viral load was 7000 IU/mL. The patient denied recent travel or animal contact. Antibodies against hepatitis A virus (HAV) and hepatitis C virus (HCV) were negative, anti-HBs antibodies were positive after reported vaccination against HBV, HBs antigen and anti-HBc antibodies were negative. Differential diagnostic assessments showed negative antinuclear, antimitochondrial, and antineutrophilic cytoplasmatic antibodies. Ion, ferritin and transferrin saturation values were within the normal range as well as coeruloplasmine.

Upon diagnosis of acute hepatitis E, immunosuppression with baricitinib was interrupted and HEV viral load and transaminase values declined further. AST was 21 (<35) U/L, ALT 16 (<35) U/L and GGT 166 (<40) U/L one week after discontinuation of baricitinib, CRP value and ESR had normalized and HEV viral load was <5000 IU/mL. HEV viral load was negative three weeks after the treatment with baricitinib had been stopped. The patient reported symptoms of arthralgia, and CRP value and ESR increased four weeks after discontinuation. Therefore, baricitinib was reintroduced into treatment. Liver enzymes and HEV viral load remained normal while CRP value and ESR normalized.

To assess HEV infection in AIRD patients treated with JAKis, we performed retrospective analysis of 138 AIRD patients which were tested for HEV RNA and anti-HEV IgM and IgG antibodies using a commercial HEV RT-PCR Kit and HEV IgG/IgM ELISA, respectively (Table S1). We identified 17 patients which were anti-HEV IgG positive, including five patients with detectable anti-HEV IgM levels (Figs. S1A and B). One patient was anti-HEV IgG negative but IgM positive. None of these six anti-HEV IgG-positive patients had detectable HEV RNA levels (data not shown), but we identified HEV ORF2 antigen in three, suggesting a recent HEV infection (Fig. S1C). Characteristics and treatment history of these three patients are summarized in Table S2. These data suggest that HEV infections occur in AIRD patients treated with JAKis. Nevertheless, the causative relationship between administration of JAKi and evidence of HEV infection needs to be addressed in larger cohorts.

3.2. JAKi therapy boosts HEV infection and progeny virus production ex vivo

Given that an AIRD patient under baricitinib therapy had hepatitis E with increased liver enzymes, CRP and ESR, we asked whether JAKis are capable to modulate HEV infection ex vivo. Therefore, we utilized the three FDA-approved JAK1 inhibitors, tofacitinib and upadacitinib as well as the PMDA-approved JAK1 peficitinib for HEV infection experiments in the human hepatoma cell line HepG2/C3A and primary human hepatocytes (PHHs). HepG2/C3A cells are a clonal derivative of liver tumor-derived HepG2 cells, whereas PHHs are isolated directly from human liver tissue. This distinct origin and genetic background results in a differential expression of numerous cellular factors in HepG2/C3A...
cells compared with PHHs. Consequently, when exposed to specific stimuli such as virus infections and drugs, HepG2/C3A cells may respond differently compared with PHHs.

First, we monitored cytotoxicity in the hepatoma cell line HepG2/C3A upon treatment with the different JAKis and detected no significant cytotoxicity (Fig. S2). Next, we infected HepG2/C3A with HEV in the presence or absence of the different JAKis. Both microscopic analysis and quantification of progeny virus production revealed that JAKi treatment did not affect HEV propagation (Fig. S2). Importantly, the absence of a JAKi-induced facilitation of HEV infection does not necessarily suggest the absence of a functional, HEV-triggered innate immune response in these cell lines.

Because of their authenticity and physiologically relevance, we next took advantage of PHH cultures for HEV infection experiments. Microscopic analyses revealed that all four JAKis lead to stronger fluorescence signal intensity in HEV-infected PHHs, whereas the guanosine analog ribavirin, used as positive control, reduced ORF2 fluorescence signal intensity (Fig. 2A). In line, quantification of progeny virus revealed increased titers with especially baricitinib leading to approx. 40-fold increased titers compared with HEV-infected, untreated PHHs (Fig. 2B). Since baricitinib caused the strongest proviral effect under all tested JAKis, we applied different doses of baricitinib (2 μM, 0.2 μM and 0.02 μM) and evaluated its effect on HEV infection at multiple time points post infection. PegIFN-α, a cognate ligand of the IFN-α/β receptor (IFNAR) and activator of JAK-STAT signaling served as a control for inhibition of HEV infection. As expected, IFN therapy potently restricted HEV infection (Fig. 2C). Our analysis further identified that baricitinib treatment facilitated HEV propagation in a dose-dependent manner, 3, 5 and 7 dpi (Fig. 2C). Of note, we observed that in both the presence and the absence of baricitinib, titers of progeny virus peaked at 3 dpi suggesting an efficient progeny virus production in the early phase of infection ex vivo.

IFN and baricitinib have reciprocal effects on the activity of the JAK-STAT signaling with JAKis acting downstream of IFN and its cognate receptor IFNAR. As a proof of principle, we performed co-treatment with IFN and baricitinib to demonstrate that the applied JAKis can revert the IFN-induced suppression of HEV infection in primary cell cultures. Single treatments confirmed that IFN restricted and baricitinib facilitated HEV progeny virus production (Fig. 2D and E). Co-treatment with baricitinib abolished the IFN-induced suppression of HEV infection and resulted in higher progeny virus titers compared with mock-treated samples. Virus titers were approx. 3-fold lower than in sole baricitinib-treated samples, indicating that IFN might induce an antiviral response by bypassing the canonical JAK-STAT pathway in our

Fig. 2. JAKis facilitate HEV infection and progeny virus production in primary human hepatocytes. (A) Representative immunofluorescence images stained for HEV ORF2 protein in HEVcc (Kernow-C1 p6) infected PHHs upon JAKi (baricitinib (BARI), tofacitinib (TOFA), upadacitinib (UPA), peficitinib (PEF)) and ribavirin (RBV) treatment. (B) Newly produced intracellular viral particles were recovered from HEVcc-infected, JAKi- or RBV-treated PHHs, 3 dpi. Lysates were used to inoculate naïve HepG2/C3A target cells. (dashed line, LOQ; titers below LOQ set to LOQ; n = 3 ± SD) (C) Newly produced intracellular viral particles were recovered from HEVcc-infected, baricitinib- or IFN-I-treated PHHs, 3, 5 or 7 dpi. PegIFN-α was applied at a concentration of 100 IU/mL and baricitinib at a concentration of 2 μM. Lysates were used to inoculate naïve HepG2/C3A target cells. (dashed line, LOQ; titers below LOQ set to LOQ; n = 3 ± SD) (D) Newly produced intracellular viral particles were recovered from HEVcc-infected, baricitinib- or IFN-I-pretreated PHHs, 3, 5 or 7 dpi. PegIFN-α was applied at a concentration of 100 IU/mL and baricitinib at a concentration of 2 μM. Lysates were used to inoculate naïve HepG2/C3A target cells. (dashed line, LOQ; titers below LOQ set to LOQ; n = 3 ± SD) (E) Newly produced intracellular viral particles were recovered from baricitinib-, IFN-I- or baricitinib/IFN-I-treated PHHs, 3 dpi. Lysates were used to inoculate naïve HepG2/C3A target cells. (dashed line, LOQ; titers below LOQ set to LOQ; n = 3 ± SD). (F) Newly produced intracellular viral particles were recovered from baricitinib-, RBV- or baricitinib/RBV-treated, HEV-infected PHHs 3 dpi. Lysates were used to inoculate naïve HepG2/C3A target cells (dashed line, LOQ; titers below LOQ set to LOQ; n = 2 ± SD).
experimental systems. As a proof of principle to evaluate whether ribavirin may be considered as antiviral treatment in HEV-infected JAKi-treated patients, we next performed co-treatment of ribavirin and baricitinib. Here, we detected significant ribavirin-caused restriction of HEV infection and progeny virus production in both presence and absence of baricitinib (Fig. 2F). These data confirm the antiviral effect of ribavirin upon inhibition of JAK-STAT signaling in the used cell culture model.

Taken together, these results suggest that JAK-dependent activation of antiviral programs is essential to control HEV infection and that perturbation of this particular signaling pathway results in facilitation of HEV infection in PHHs.

3.3. RNA sequencing reveals an accumulation of HEV RNA in the presence of baricitinib

The JAK-STAT pathway plays a pivotal role to mount a robust antiviral response upon virus encounter, and the resulting antiviral state within the cells is based on the induction of interferon-stimulated gene (ISG) expression. As shown in Fig. 2, inhibition of this pathway by JAKi treatment promoted the HEV replication cycle. To elucidate whether baricitinib treatment affects the steady-state gene expression in the absence of pathogen encounter as well as in presence of HEV, we performed transcriptomic analysis. PHH cultures were either treated with baricitinib (2 μM or 0.2 μM), infected with HEV or baricitinib-treated and HEV-infected. Total RNA was extracted and supplied to Illumina RNAseq 3 dpi. First, we evaluated the viral transcriptome. In the presence of baricitinib, the number of reads that mapped to the viral reference genome Kernow-C1 p6 (GenBank accession no. JQ679013) were approx. 16-fold (for 0.2 μM) and 34-fold (for 2 μM) higher compared with untreated samples (Fig. 3A). These data highlight the proviral effect of baricitinib treatment on HEV infection. Under all conditions, HEV transcripts mapping to the ORF2/ORF3 region were more abundant than transcripts mapping to ORF1 (Fig. 3B). Those observations are based on the fact, that during HEV replication not only a full-length viral genome is produced, but also a 2.2 kb subgenomic transcript encoding for the structural ORF2 and ORF3 proteins, resulting in higher numbers of transcripts mapping to HEV ORF2/ORF3 than to ORF1. Given that the HEV virus replication cycle is error-prone and considering that baricitinib increase HEV replication, we asked whether application of the JAKis enhanced the generation of quasispecies in our experimental setting. Hence, we analyzed single nucleotide variant (SNV) frequencies in baricitinib-compared with mock-treated samples by aligning viral reads to the genome of the utilized Kernow-C1 p6 strain. As shown in Fig. S3, SNV frequencies were not elevated compared with untreated specimens, suggesting that application of baricitinib does not increase viral diversity in the early phase of infection in PHH cell culture systems.

3.4. Baricitinib efficiently inhibits the HEV-triggered upregulation of ISGs in PHH cultures

Next, we aimed to decipher how baricitinib treatment and HEV infection modulate the transcriptional host response in PHHs. For this, we mapped RNAseq data to the hg38 genome scaffold and normalized raw data allowing to analyze gene expression levels. Principal-component analyses (PCAs) revealed a separation of mock-treated/mocked-infected, baricitinib-treated/mock-infected, mock-treated/HEV-infected and baricitinib-treated/HEV-infected samples. These analyses already revealed that especially baricitinib treatment and HEV infection resulted in a distinct global gene expression profiles (Fig. 4A). Within the baricitinib-treated samples, the effect of the differential concentrations was only modest.

Further, we observed a deregulation of a substantial number of genes (reads per kilobase million [RPKM] ≥ 0.5 and fold change ≥ 2) under all tested conditions, indicating that both HEV infection and baricitinib treatment affect the transcriptome in PHHs (Fig. 4B). Baricitinib treatment resulted rather in depression of gene expression including the proinflammatory cytokine CXCL2 (C-X-C motif chemokine ligand 2), whereas HEV infection mobilized gene expression, dominated by the upregulation of interferon-stimulated genes including IFTT2 (interferon-induced protein with tetratricopeptide repeats 2), CXCL10 and MX1 (Fig. S4). Gene ontology (GO) enrichment analyses of biological processes revealed the modulation of antiviral, immune and metabolic responses. Here, the regulation of immune system processes was especially involved upon HEV infection (Fig. 4C). We next evaluated the regulation of individual GO-terms and detected ‘inhibition of type I IFN signaling’, ‘response to virus’ and ‘response to cytokines’ in the presence of baricitinib and/or HEV infection (Fig. 4C). Importantly, treatment with 2 μM baricitinib (and to a lesser extent 0.2 μM baricitinib) restricted the HEV-induced activation of these pathways. Taken together, our analyses suggest a dose-dependent and reciprocal effect of baricitinib and HEV infection on multiple immune associated pathways.

![RNAseq of HEV-infected PHHs](image)
We next aimed to decipher expression levels of individual genes associated with proinflammatory cytokine/chemokine production and signaling. We observed a HEV-triggered induction of several genes, with especially CXCL11, CXCL10 and different IFN genes being upregulated (Fig. S4). Interestingly, the HEV-induced upregulation of some, but not all cytokine genes was dampened by baricitinib treatment. Most prominent, interferon III (IFN-III) gene expression was still induced in the presence of baricitinib, whereas induction of IFN-I genes was restricted (Fig. S4). These data imply that the HEV-induced expression of IFN-I but not IFN-III might be mediated via baricitinib-sensitive signaling pathways.

Since IFN production and signaling result in the induction of ISG expression, we next examined whether baricitinib treatment and HEV infection modulate the expression of ISGs. For the following analysis, we focused on the evolutionary conserved collection of “mammalian core ISGs” that are expressed in the majority of mammalian species. It comprises genes encoding proteins involved in antigen presentation, IFN induction and suppression, cell signaling, ubiquitination and protein degradation, thereby condensing important parts of the innate immune response in a single gene set (Shaw et al., 2017). Of note, baricitinib treatment alone caused down-regulation of multiple core ISGs, pointing towards a reduced antiviral capacity under steady-state conditions. Here, especially expression of STAT1 and IRF9 (interferon regulatory factor 9), two integral components that mediate resistance to viruses, was decreased (Fig. 5A and B) (Cheon et al., 2013). HEV encounter induced an antiviral state illustrated by the upregulation of multiple cytokines and ISGs (Fig. 5A–C, Fig. S4). Baricitinib treatment of PHHs dampened this HEV-triggered upregulation for the majority of ISGs. We observed that 2 μM baricitinib could fully inhibit the HEV-triggered upregulation of ISG expression, whereas 0.2 μM baricitinib dampened
the upregulation (i.e. IFITM1 (interferon-induced transmembrane protein 1)) (Fig. 5B–C). For a subset of ISGs, baricitinib did not affect the HEV-caused induction of gene expression (i.e. RSAD2 (Radical S-adenosyl methionine domain-containing protein 2)) (Fig. 5B–C). These data imply a differential relevance of the JAK-STAT pathway for expression and its modulation of distinct ISG subsets in the context of HEV infection.

In summary, the gathered data indicate that (1) baricitinib treatment downregulates genes which are critical for a robust antiviral response, (2) HEV induces a broad innate immune response in PHHs and (3) baricitinib inhibits the HEV-triggered immune response and thereby facilitates HEV infection.

4. Discussion

In the last decade, selective JAKis have been approved for the treatment of moderate to severe rheumatoid arthritis (Keystone et al., 2015). Given the immunomodulatory nature of JAKis, multiple clinical-trial and real-world studies of JAKis suggest an increased risk of treatment emergent infections and reactivation of latent viruses (Winthrop et al., 2020). I.e., the risk of herpes zoster was 1.5–2-fold higher than those usually observed in patients with RA (Winthrop et al., 2014). Recent evidence added another perspective with a more ambivalent role of JAKis and viral infections: especially baricitinib came into spotlight as a potentially therapy against SARS-CoV-2, because its immunosuppressive effect may mitigate disease severity by limiting dysregulation of the immune system such as hypercytokinemia (Hoang et al., 2021).

Given the ambivalent role of JAKis for SARS-CoV-2 and especially the potential risk of JAKi treatment for emergent viral infections, we aimed to assess HEV infection in AIRD patients treated with JAKis. We identified and evaluated the clinical course of a HEV RNA positive AIRD patient with elevated liver enzymes. Subsequent systematic monitoring of AIRD patients revealed that HEV seroprevalence in AIRD patients treated with JAKis was in the predicted range for the general population in Europe (Hartl et al., 2016). In this cohort, we detected HEV antigen in three patients with anti-HEV IgM antibodies, suggesting a recent HEV infection. Since HEV is a leading cause of acute viral hepatitis worldwide, additional safety measures, including screening for HEV RNA and especially education about HEV transmission might be considered for AIRD patients.

Limitations of the cohort are a relatively small size and that longitudinal samples and samples prior to the beginning of JAKi treatment were not available. Additionally, the cohort only consists of patients either diagnosed with RA or psoriatic arthritis. Despite these limitations, it’s the first cohort of JAKi-treated patients which was analyzed for parameters of a HEV infection in a real-world setting.

JAK-STAT pathway is a central communication node regulating both innate and adaptive immunomodulatory processes (Villarino et al., 2017). In this study, we aimed to focused on the innate immunity part and took advantage of in vitro/ex vivo models allowing to largely exclude adaptive immune processes. Although hepatoma cell lines are a suitable model to study multiple aspects of HEV virology, they often bear the limitation of potentially impaired innate immune cascades (Sumpter et al., 2005). Hence, we utilized PHH cultures in this study with the goal to address our hypothesis in one of the most authentic cell culture models.

Our experimental ex vivo data with PHH cultures show that the innate immunity is critical to control HEV infection and that blunting of the innate immunity by JAKi treatment can lead to an immense increase of HEV replication (Figs. 2 and 3). The restriction of HEV is probably exerted by ISGs, which form the antiviral effectors of the innate immunity. However, no ISGs with anti-HEV activity have been reported so far. In addition to HEV, facilitation of viral life cycle progression has been observed for HCV but not HBV in PHH cultures upon
pharmacological JAK inhibition (Shen et al., 2018; Tegtmeier et al., 2021). Combinatory treatment of the two opposing drugs IFN-β and baricitinib revealed that baricitinib could reverse the antiviral effect of IFN. However, we further observed that IFN restricted HEV infection in baricitinib-treated PHHs. This reduction may point to a contribution of non-canonical IFNAR signaling to the antiviral response in PHHs.

The transcriptome analyses underscored that baricitinib treatment in the absence of pathogen encounter resulted in a downregulation of multiple ISGs, pointing towards a lowered antiviral capacity under steady state conditions and a contribution of JAK-STAT signaling to baseline immunity. HEV infection caused the induction of antiviral programs, and baricitinib-treatment promoted HEV infection concomitant with a reduced upregulation of pro-inflammatory cascades (Figs. 4 and 5).

This data highlight the importance of a robust innate immune response to control HEV infection in PHHs and the contribution of the JAK-STAT pathway to maintain baseline immunity. Intriguingly, the transcripts further provide evidence that ISG expression profiles might be clustered with regard to their dependency on the JAK-STAT pathway.

In conclusion, we evaluated the effect of JAKi therapy on HEV infection. Ex vivo analyses revealed a 20× increase of HEV replication in the presence of baricitinib. Although it is not clear whether baricitinib promoted HEV infection in the reported case and the role of adaptive immunity to control HEV in vivo still has to be defined, our findings raise potential concern given that innate immunity is orchestrated via JAK-STAT signaling. Therefore, patient briefing about risk factors of HEV transmission and establishment of rational safety measures such as screening of HEV RNA prior to start and in case of elevated liver enzymes during JAKi therapy should be considered.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2023.105690.

References


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Data availability

Data will be made available on request.

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