Isolation and characterization of interferon lambda-resistant hepatitis C virus replicon cell lines

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**ABSTRACT**

Pegylated interferon lambda-1a (Lambda) is currently in clinical development for the treatment of chronic hepatitis C virus (HCV) infection. To gain insight into the potential mechanisms of non-responsiveness that may occur in patients treated with Lambda, HCV subgenomic replicon cell-lines with impaired susceptibility to the unpegylated recombinant (r) form of interferon (IFN) lambda-1 (rIFNλ) were isolated and characterized. The selected replicon cell populations showed a defect in the activation of the IFN-dependent JAK-STAT signaling pathway. Reduced phosphorylation of STAT proteins and lower expression levels of the cellular janus kinases Jak1 and Tyk2 were observed in these cell populations, which may account for the impaired JAK-STAT signaling and reduced antiviral responses to rIFNλ. Overall, this in vitro study provides molecular insights into the possible mechanism of viral evasion to rIFNλ in the HCV replicon cell system.

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**Introduction**

Hepatitis C virus (HCV) is a major etiologic agent of liver disease, afflicting an estimated 170 million people worldwide and resulting in approximately 350,000 deaths annually (World-Health-Organization, 2011). Over the last decade, a combined regimen of pegylated interferon alfa (alfa) and ribavirin (RBV) has been the backbone of therapy for the management of chronic HCV infection. Unfortunately, a significant proportion of patients fail to respond or relapse after cessation of treatment. Genetic variations and viral determinants are believed to influence the outcome of infection, and different HCV genotypes have been associated with variable responses to alfa-RBV regimens (Fried et al., 2002; Ghany et al., 2009; Hayes et al., 2012). Sustained virologic response (SVR) is achieved in 40–50% of patients infected with the most predominant HCV genotype (GT) 1 (subtypes 1a and 1b), whereas higher SVR rates (78–86%) have been reported in patients infected with HCV GT2 and GT3. The recent introduction of direct acting antiviral (DAA) agents targeting the HCV NS3 protease has dramatically improved SVR rates and shortened treatment durations, particularly for patients infected with GT1, (Ghany et al., 2011) Inclusion of either NS3 protease inhibitors (NS3 PIs) boceprevir or telaprevir to the alfa-RBV regimen has resulted in 70–75% SVR rates in previously untreated GT1 patients (Poordad et al., 2011; Jacobson et al., 2011). However, non-responsiveness to alfa continues to be a substantial hurdle to more effective and tolerable IFN-based therapies.

Human IFN lambda-1 (IFNλ1), also known as interleukin-29 (IL-29), IFNλ2 (IL-28A) and IFNλ3 (IL-28B) are newly described type III IFNs which have a close evolutionary relationship to the interleukin-10 (IL-10) cytokine family, and are distantly related to the type I IFNs (Kotenko et al., 2003). The biological characteristics of these cytokines are comparable to those of a type I IFN, such as IFN-alpha (IFNα) and IFN-beta (IFNβ), although there is limited sequence homology (Donnelly and Kotenko, 2010). Similar to IFNα/β, expression of IFNλ1 is induced upon viral infection, and has demonstrated broad antiviral activity in numerous HCV replication cell-based models (Marcello et al., 2006; Marukian et al., 2011; Friborg et al., 2013). These various classes of IFN exert their antiviral activities by inducing the expression of IFN-stimulated genes (ISGs). Signal transduction through engagement of distinct heterodimeric receptor complexes lead to common activation of the janus kinases, Jak1 and Tyk2, and subsequent phosphorylation of the signal transducer and activator of transcription (STAT) factors, STAT1 and STAT2. In the type I IFNα receptor (R) complex, the IFNAR1 and IFNAR2 chains contribute to ligand binding and activation of Tyk2 and Jak1, respectively. Conversely, type III IFNs receptor complex composed of the ubiquitously expressed IL-10Rβ chain (also called IL-10R2) and the unique IL-28Rα chain play comparable roles to the IFNα co-receptor subunits IFNAR1 and IFNAR2. Activated STAT1 and STAT2
recruit the IFN regulatory factor 9 (IRF9) to form the trimeric IFN-stimulated gene factor 3 (ISGF3) complex competent for nuclear translocation and subsequent binding to the IFN-stimulated response element (ISRE) located upstream of various ISGs. The complete spectrum of ISGs that mediate an antiviral effect on HCV replication has not yet been defined (Schooggins et al., 2011).

Given the comparable biological properties of these two families of cytokines, it is important to understand whether type III IFNs could provide an alternative treatment option to alfa with improved SVR rates. To this end, the investigational pegylated form of IFNα1 (Lambda), has demonstrated efficacy in subjects infected with HCV GT1 through GT4 (Zeuzem et al., 2011, 2012). Data from a Phase 2b clinical study indicated that SVR rates at 24 weeks after completing treatment were comparable for Lambdab-RBV and alfa-RBV in previously untreated patients with HCV GT1 or GT4 (Muir et al., 2012). However, the Lambda regimen produced a more rapid decline in HCV RNA through 12 weeks of treatment compared to the alfa regimen. Thus, a better understanding of the contrasting responses associated with Lambda would undoubtedly help in improving outcomes of IFN-based therapy.

The precise mechanisms underlying treatment failure and limited responsiveness to alfa-based therapy are not fully understood, but various reports have implicated the contribution of multiple host and viral factors. Asselah et al. (2010) Fundamental insights into mechanisms of resistance to IFNα have been established on studies performed in the HCV replicon system (Namba et al., 2004; Hazari et al., 2007; Noguchi et al., 2008; Datta et al., 2011). This human hepatoma cell-culture model carrying self-replicating HCV subgenomic RNAs representing various GTs has provided a powerful tool for identifying novel classes of HCV inhibitors, as well as being useful in understanding their resistance development profiles (Gao et al., 2010; McPhee et al., 2012b). Consequently, studies described in this report were initiated to establish on studies performed in the HCV replicon system (Namba et al., 2004; Hazari et al., 2007; Noguchi et al., 2008; Datta et al., 2011). This human hepatoma cell-culture model carrying self-replicating HCV subgenomic RNAs representing various GTs has provided a powerful tool for identifying novel classes of HCV inhibitors, as well as being useful in understanding their resistance development profiles (Gao et al., 2010; McPhee et al., 2012b). Consequently, studies described in this report were initiated to investigate the potential of HCV resistance to IFNα in vitro using the replicon cell system, and to define its underlying molecular basis. Overall, our results suggest that a defect in the JAK-STAT signaling pathway blocks the IFN-induced gene activation, causing decreased susceptibility to rIFNα in the HCV replicon cell-based model.

**Results**

**Generation of IFN-resistant HCV replicon cell lines**

The HCV replicon cell-based system is a useful tool for characterizing resistance to antiviral replication inhibitors and has so far been predictive of the emergence of resistance variants to DAA in clinical development. Indeed, non-clinical studies have documented the emergence of several amino acid substitutions in the NS5 protease region using various HCV replicon cell-based models. One of these variants is R155K and has been associated with resistance to the recently approved NS3 PIs boceprevir and telaprevir in patients failing treatment (Bartels et al., 2008; Susser et al., 2011). Therefore, to investigate the molecular mechanisms underlying resistance to Lambda treatment, HCV subgenomic replicon cell lines representing a wild-type (WT) GT1a or a GT1a variant encoding R155K in the NS3 domain were maintained in parallel under selective pressure with either rIFNα or rIFNα at 30- or 100-times their EC50 values, and in the presence of G418. Low multiple EC50 values were applied for long-term treatment to specifically avoid curing of the cells from the HCV replicon, as previously reported (Lemm et al., 2005). These HCV replicons contain an in-frame gene encoding the neomycin phosphotransferase (neoR) gene conferring resistance to G418. Replicons that are not capable of replication under specific compound inhibitory pressure will not retain their capacity to rescue host cell viability through neomycin resistance. Accordingly, visual inspection of the cultures under IFN selective pressure indicated reduced growth rates and widespread cell death during the first week of treatment. Following prolonged passages (> 60 days) under selective pressure in the presence of G418, cells emerged from the initial dying population indicating the presence of HCV replicons that were refractory to IFN inhibition. In the GT1a selection, cell cultures exposed to 100 times EC50 multiples of test compounds showed significant growth impairment and they were therefore excluded from further analysis. Breakthrough GT1a IFN-resistant cell populations selected using 30 times EC50 multiples of test compounds were phenotyped for their susceptibility to various classes of HCV replication inhibitors. As shown in Table 1, the GT1a replicon cell population exposed to rIFNα exhibited reduced susceptibility to the cytokine (6-fold over WT control level), while retaining sensitivity to the inhibitory effects of rIFNα. In contrast, GT1a replicon cells selected in the presence of rIFNα showed reduced susceptibility to both rIFNα (3-fold) and rIFNα (6-fold) relative to the parental control cells. Fold resistance was greater in the GT1a NS3-R155K replicon cell selected in the presence of 30 times EC50 multiples of rIFNα. Indeed, the NS3-R155K breakthrough IFNα-resistant replicon cell population demonstrated 6- and 12-fold reduced susceptibility to rIFNα and rIFNα, respectively, relative to the parental control cells. The NS3-R155K breakthrough IFNα-resistant cell populations selected at 30 and 100 times the IFNα EC50 values, conferred higher resistance to rIFNα (7- and 19-fold, respectively) while having low levels of resistance to rIFNα (4-fold). More importantly, all IFNα-resistant cell lines tested retained susceptibility towards the HCV NS3 PI asunaprevir relative to parental control replicon cell lines suggesting that the resistance phenotypes were specific to IFN treatments (Table 1).

**Table 1**

Antiviral activity of HCV replication inhibitors against GT1a WT and NS3-R155K replicons selected during treatment with rIFNα or rIFNα.

<table>
<thead>
<tr>
<th>Cell line (GT1a)</th>
<th>Compound EC50 (fold resistancea)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASV (µM)</td>
</tr>
<tr>
<td>WT control</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>WT IFNα (30 × 1)</td>
<td>0.007 ± 0.001 (1)</td>
</tr>
<tr>
<td>WT IFNα (30 × 1)</td>
<td>0.005 ± 0.001 (1)</td>
</tr>
<tr>
<td>NS3-R155K control</td>
<td>0.546 ± 0.061</td>
</tr>
<tr>
<td>NS3-R155K IFNα (30 × 1)</td>
<td>0.401 ± 0.096 (1)</td>
</tr>
<tr>
<td>NS3-R155K IFNα (30 × 1)</td>
<td>0.466 ± 0.096 (1)</td>
</tr>
<tr>
<td>NS3-R155K IFNα (100 × 1)</td>
<td>0.356 ± 0.063 (1)</td>
</tr>
</tbody>
</table>

Values are the average ± standard deviation from ≥3 independent experiments.

ASV = asunaprevir; 1453 = BVDV specific inhibitor.

a Fold over parental control cells is calculated from individual experiments and reported as an average.
To determine whether amino acid substitutions in the HCV replicon genome were responsible for the IFN-resistance phenotypes, genotypic sequencing analysis of the selected replicon cell populations was performed and compared to their respective parental control cell lines. Total RNA was extracted and cDNA subjected to population sequencing over the entire coding region of the HCV replicon genome. As summarized in Table 2, none of the replicon cell lines selected under rIFNα pressure showed identical genomic sequences with multiple point mutations emerging throughout the NS3, NS5A and NS5B coding regions of the various HCV genomes. None of the mutation sites overlapped with a region in NS5A (encompassing amino acids 237–276) referred to as the IFN sensitivity determining region (ISDR). HCV variants with mutations in this putative ISDR central region of NS5A were previously reported to influence the outcome of IFN therapy, suggesting a role for the HCV protein in IFN resistance (Nakagawa et al., 2010; Tran et al., 2011). No amino acid substitutions were identified in the NS4A and NS4B coding regions of the various IFN-resistant replicon cell lines. Taken together, our initial findings suggest that HCV viral factors are not involved in the mechanism of IFNα resistance in these replicon cells.

### Susceptibility profiles against bovine viral diarrhea virus replication in IFN-resistant replicon cells

To further investigate the contribution of viral factors in the mechanisms of rIFNα resistance, susceptibility profiles to IFN treatment were evaluated against the related but genotypically distinct BVDV replicon (O’Boyle et al., 2005). Replication of a BVDV replicon carrying an in-frame firefly luciferase gene was monitored in the GT1a NS3-R155K IFNα-resistant replicon cell population. As shown in Table 3, replication of the BVDV replicon transiently expressed in the sensitive and IFN-resistant replicon cell lines was equally suppressed by a selective BVDV inhibitor, compound 1453 (Sun et al., 2003). Accordingly, asunaprevir was inactive (EC50 > 5 μM) against replication of the BVDV replicon in cells. In contrast, loss of potency to both rIFNα and rIFNλ against replication of BVDV replicon was significant in the IFNα-resistant cell lines (14-fold and 8-fold over parental control cells, respectively). As seen during the HCV phenotypic profiling, cells selected in the presence of rIFNα were 11- to 12-fold less susceptible to this cytokine while demonstrating only modest 4- to 5-fold losses in potency to rIFNλ, suggesting reduced cross-resistance to this treatment. The similarity in the resistance phenotypic profiles against HCV replication or BVDV replication in these IFN-selected replicon cell populations strongly suggest that a common defect in host-related pathways is responsible for the decreased susceptibility reported against rIFNα and rIFNλ, rather than selection of viral drug-resistant variants.

### Reduced ISRE signaling in IFN-resistant replicon cell lines

To understand the significance of the reduced susceptibility phenotypes, we next performed studies to determine the functionality of the JAK-STAT signaling pathway in these IFN-resistant cell populations. Activation of the IFN-mediated signaling pathway in replicon cells was monitored using a luciferase-based reporter construct under the control of the ISRE promoter. Cells transiently expressing the ISRE-luciferase reporter construct were exposed to various concentrations of rIFNα (1–5000 IU/ml) or rIFNλ (1–2500 ng/ml) for 24 h. Luciferase activity was then measured in these cell lysates and results expressed as fold activation over untreated cells. As shown in Fig. 1, the levels of ISRE-promoter activation for the different IFNα- and IFNλ-resistant cell populations correlated with the IFN susceptibility profiles. Reduced activation of the ISRE-promoter in the NS3-R155K IFNα-resistant...
Table 3
Antiviral activity of rIFNα against bovine viral diarrhea virus (BVDV) replicon transiently replicating in the IFN-resistant HCV cell lines.

<table>
<thead>
<tr>
<th>Cell line (GT1α)</th>
<th>Compound EC₅₀ (fold resistance*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASV (μM)</td>
</tr>
<tr>
<td>NS3-R155K control</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>NS3-R155K IFNα* (30 ×)</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>NS3-R155K IFNλ* (30 ×)</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>NS3-R155K IFNλ* (100 ×)</td>
<td>&gt; 5</td>
</tr>
</tbody>
</table>

Values are the average ± standard deviation from ≥3 independent experiments.
* Fold over parental control cells is calculated from individual experiments and reported as an average.

Fig. 1. Stimulation of the IFN signaling pathway in GT1α NS3-R155K replicon cell lines sensitive and resistant to rIFNα. GT1α NS3-R155K IFNα (A) and GT1α NS3-R155K IFNλ (B) resistant cell lines were transfected with the pISRE-Luc reporter plasmid, and induction profiles compared in parallel to the sensitive parental cell line. The activation of the ISRE promoter in the sensitive and IFNλ-resistant replicon cell lines was determined in the presence or absence of increasing concentrations of rIFNα (1–5000 IU/ml) or rIFNλ (1–2500 ng/ml) 24 h following stimulation. Fold activation values represent the average from ≥3 independent experiments.

Defective JAK-STAT signaling pathway in IFN-resistant replicon cells

To gain insights into the mechanisms of the defective IFN signaling in the resistant replicon cell lines, expression levels of various cellular components involved in the JAK-STAT pathway were monitored upon stimulation with rIFNα or rIFNλ, and compared to the control parental cell line. GT1α NS3-R155K sensitive and resistant replicon cell populations were exposed to rIFNα or rIFNλ at different concentrations, and cell lysates harvested following 30 min stimulation. Equal amounts of protein extracts were subjected to Western immunoblot analysis using antibodies directed against the phosphorylated or un-phosphorylated forms of STAT1 and STAT2. These findings suggest that a defect up-stream of the cellular JAK-STAT signaling pathway may be responsible for the observed IFN-resistant phenotypes in replicon cells.
rIFNα- and rIFNλ-resistant cell lines (Fig. 3). Results from the Western immunoblot analysis indicated that both IFNα- and IFNλ-resistant replicon cell lines have reduced expression of Tyk2 as compared to the control parental cell line. The expression of Jak1 was also reduced in the IFNλ-resistant cell population, while not being affected in the IFNα-resistant cell population when compared to the control parental cell line.

Tyk2 was previously shown to stabilize the cell surface expression of IFNAR1 by decreasing its basal internalization rate (Ragimbeau et al., 2003; Marijanovic et al., 2006). In the absence of Tyk2, IFNAR1 localized primarily into perinuclear endosomal compartments, potential sites of proteolysis. To assess whether the reduced expression of Tyk2 noted in the IFN-resistant replicon cells influenced the localization of IFNAR1, indirect immunofluorescence staining analysis was performed in these cells (Fig. 4). As previously reported (Ragimbeau et al., 2001), abundant distribution of Tyk2 was observed in both the cytoplasmic and the nuclear compartments of the parental IFN-sensitive cell line (panel a). In accordance, staining of the IFNAR1 in those cells revealed both a cytoplasmic and plasma membrane distribution (panels d and g). Conversely, the intracellular staining of Tyk2 was very weak in both IFN-resistance replicon cell population (panels e and h). Staining of IFNAR1 in the IFNα-resistant cell population was found to be localized in the perinuclear endosomal compartment (panels e and h). On the other hand, IFNAR1 staining in the IFNλ-resistant cell populations was predominantly localized in the perinuclear space (panels f and i). In the IFNα heterodimeric receptor complex, Tyk2 interacts with the IL-10Rβ chain. However, little is known about the effect of Tyk2 on the cellular distribution of IL-10Rβ. Staining of IL-10Rβ in the sensitive parental cell line revealed a punctuate cytoplasmic distribution (panels j and m). A similar cellular pattern was detected in the IFNα-resistant cell population. In contrast, a dense perinuclear punctuate IL-10Rβ staining was noted in the IFNα-resistant cell population (panels l and o). The overall protein synthesis of cognate subunits of IFNα and IFNλ receptor complexes was not affected in the resistant cell.
ified populations as documented by quantitative real-time PCR and Western immunoblot analysis (see Fig. S3 in the supplemental material). Taken together, these data suggest that the reduced Tyk2 expression in cells altered the localization pattern of the IFNAR1 and IL-10Rβ subunits.

Reduced expression of genes related to the JAK-STAT signaling pathway in IFN-resistant replicon cells

In light of differences in activation of the JAK-STAT pathway observed in the IFN-resistant replicon cell populations, expression of related signal transduction genes was examined using a cDNA microarray analysis (RT2 profiler array). In these experiments, sensitive and resistant replicon cell populations were stimulated for 24 h with rIFNα (100 IU/ml) or rIFNλ (10 ng/ml), while control cells were left untreated. Following IFN stimulation, total RNA was purified from cells and gene expression profiling for human JAK-STAT target components was performed using the RT2 profiler PCR arrays. To analyze the direct effects of IFN resistance, we compared gene expression profiles between the control parental cell line and each of the IFN-resistant cell populations. As shown in Table 4, 10 genes were up-regulated (≥3-fold cut-off range) following rIFNλ treatment in the sensitive GT1a NS3-R155K cell line. These genes included known ISGs and chemokines induced by STAT1 (CXCL9, GBP1, JUNB, NOS2, ISG15, OAS1), as well as transcription factors that interact with STAT proteins (IRF1, IRF9); previously reported to be up-regulated upon rIFNλ stimulation in HCV replicon models (Marcello et al., 2006).

Fig. 4. Reduced expression of Tyk2 alters subcellular localization of IFNAR1 in IFN-resistant replicon cells. Distribution of Tyk2, IFNAR1 and IL-10Rβ was monitored by fluorescence microscopy in the GT1a NS3-R155K replicon cell lines that were sensitive or resistant to IFN treatments. Cells were fixed, permeabilized and stained with an antibody to Tyk2, IFNAR1, or IL-10Rβ, followed by (red) Alexa Fluor 594- and (green) Alexa Fluor 488-coupled secondary antibodies, respectively. Nuclei were counterstained with (blue) Hoeschst dye as shown in the overlays (panels: a–c, g–i and m–o).

Table 4
Overview of genes up-regulated following induction by rIFNα or rIFNλ in IFN-resistant GT1a replicon cell lines.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene symbol</th>
<th>Description</th>
<th>NS3-R155K</th>
<th>NS3-R155K IFNα</th>
<th>NS3-R155K IFNλ</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_002416</td>
<td>CXCL9</td>
<td>Chemokine (C-X-C motif) ligand 9</td>
<td>7.7</td>
<td>7.9</td>
<td>&lt; cutoff&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NM_005101</td>
<td>ISG15</td>
<td>ISG15 ubiquitin-like modifier</td>
<td>40.6</td>
<td>90.8</td>
<td>18.3</td>
</tr>
<tr>
<td>NM_002053</td>
<td>GBP1</td>
<td>Guanylate binding protein 1, interferon-inducible</td>
<td>5.1</td>
<td>12.1</td>
<td>&lt; cutoff&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NM_002198</td>
<td>IRF1</td>
<td>Interferon regulatory factor 1</td>
<td>&lt; cutoff&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3</td>
<td>&lt; cutoff&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NM_006084</td>
<td>IRF9</td>
<td>Interferon regulatory factor 9</td>
<td>11.8</td>
<td>14.5</td>
<td>11.7</td>
</tr>
<tr>
<td>NM_000625</td>
<td>NOS2</td>
<td>Nitric oxide synthase 2, inducible</td>
<td>3.7</td>
<td>4.8</td>
<td>&lt; cutoff&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NM_002534</td>
<td>OAS1</td>
<td>2'-5'-oligoadenylate synthetase 1, 40/46kDa</td>
<td>7.4</td>
<td>23.6</td>
<td>5.7</td>
</tr>
<tr>
<td>NM_007315</td>
<td>STAT1</td>
<td>Signal transducer and activator of transcription 1, 91kDa</td>
<td>&lt; cutoff&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4</td>
<td>&lt; cutoff&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NM_005419</td>
<td>STAT2</td>
<td>Signal transducer and activator of transcription 2, 113kDa</td>
<td>&lt; cutoff&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8</td>
<td>&lt; cutoff&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fold decrease in gene expression is calculated for each cell population over control parental GT1a NS3-R155K cell line.

<sup>b</sup> An arbitrary cutoff of ≥3-fold reduction over baseline was applied.

In light of differences in activation of the JAK-STAT pathway observed in the IFN-resistant replicon cell populations, expression of related signal transduction genes was examined using a cDNA microarray analysis (84 target genes included in the RT2 profiler array). In these experiments, sensitive and resistant replicon cell populations were stimulated for 24 h with rIFNα (100 IU/ml) or rIFNλ (10 ng/ml), while control cells were left untreated. Following IFN stimulation, total RNA was purified from cells and gene expression profiling for human JAK-STAT target components was performed using the RT2 profiler PCR arrays. To analyze the direct effects of IFN resistance, we compared gene expression profiles between the control parental cell line and each of the IFN-resistant cell populations. As shown in Table 4, 10 genes were up-regulated (≥3-fold cut-off range) following rIFNα treatment in the sensitive GT1a NS3-R155K cell line. These genes included known ISGs and chemokines induced by STAT1 (CXCL9, GBP1, JUNB, NOS2, ISG15, OAS1), as well as transcription factors that interact with STAT proteins (IRF1, IRF9); previously reported to be up-regulated upon rIFNλ stimulation in HCV replicon models (Marcello et al., 2006).
There were differences in the number of genes up-regulated and the magnitude of their stimulation in the rIFNα- and rIFNλ-resistant cell populations when compared to the sensitive parental cell line, correlating with a defect in the JAK-STAT signaling pathway. The gene most strongly induced by both cytokines was ISG15 which increased by 41-fold and 91-fold in the presence of rIFNα and rIFNλ, respectively. However, the level of induction of ISG15 mRNA expression was lower in the rIFNα- (22-fold) and rIFNλ- (29-fold) resistant cell populations upon rIFNα stimulation. Expressions of other modulated genes were also markedly reduced in the IFN-resistant cell populations compared to the IFN-sensitive parental cell line. These data support the notion that a failure to activate the JAK-STAT signaling pathway in these cell populations contributes to the in vitro mechanism of IFNα resistance.

Discussion

The molecular mechanisms underlying variable responses to alfa treatment remain poorly understood. However, several reports have suggested that viral and host factors may contribute to the lack of efficacy to alfa during HCV infection (Asselah et al., 2010; Doyle et al., 2012). In this report, we utilized the HCV subgenomic replicon system representing GT1a for the generation and characterization of cells with reduced susceptibility to rIFNα treatment. Our overall findings document for the first time that alteration in the host-related JAK-STAT signaling pathway during long-term exposure to low doses of rIFNα may lead to viral evasion.

Phenotypic analysis demonstrated that the susceptibility of the breakthrough HCV replicons under selective pressure with rIFNα significantly decreased against this cytokine, while retaining susceptibility to rIFNλ. In contrast, replicon cells selected in the presence of rIFNλ demonstrated reduced susceptibility to both cytokines relative to parental control cells. Similar phenotypic profiles were noted against a GT1a replicon cell line encoding NS3 protease R155K, a variant that has been shown to confer cross-resistance to the two NS3 PIs boceprevir and telaprevir. Resistance profiles were specific to IFN treatments as selected replicon cell populations and parental control cell lines showed comparable susceptibilities to the NS3 PI asunaprevir (Table 1) and the NS5A replication inhibitor daclatasvir (data not shown). Moreover, sequence analysis of IFNλ-resistant replicon cell populations failed to identify common amino acid substitutions in the HCV genome responsible for the resistance phenotypes. Sequence variations within a discrete region of NS5A defined as the IFN sensitivity determining region (ISDR) have been associated with negative outcomes to alfa treatment (Enomoto et al., 1996; Hayashi et al., 2013). However, conflicting results over the years, especially from studies investigating European and North American HCV isolates, have questioned this correlation (Tan and Katze 2001). Our genotypic analysis of the selected replicon revealed the emergence of random point mutations throughout the NS3, NS5A and NS5B coding regions of the various HCV genomes. None of the mutation sites overlapped with the NS5A ISDR domain suggesting a lack of association with the IFN resistance phenotypes described in the replicon cell populations. More importantly, replication of the related but genotypically distinct BVDV replicon transduced in these selected cell populations demonstrated similarly reduced susceptibilities to rIFNλ and rIFNα inhibition. Based on these collated data, mechanisms of rIFNα resistance in these cell populations appear to involve altered host pathways rather than direct interactions with viral factors.

To further define the reduced susceptibility profiles established in the IFN-resistant cell populations, the functionality of the IFN signaling pathway was investigated next by monitoring the activation of a reporter luciferase construct driven by the ISRE promoter upstream of IFN responsive genes. All IFNλ-resistant replicon cell lines had reduced levels of ISRE-promoter activation in line with their viral phenotypic profiles. The IFNα-resistant replicon cell populations showed reduced ISRE-driven luciferase activity after stimulation with rIFNα or rIFNλ when compared to the parental control cell lines. In contrast, minimal cross-resistance to rIFNλ was noted in the IFNλ-resistant replicon cell population which corroborated with the viral phenotypic profile. These disparate cross-resistance profiles are intriguing in light of studies documenting the establishment of a refractory state of the IFN transduction pathway in various cell lines and in mice upon repeated stimulation with rIFNα (Sarasin-Filipowicz et al., 2009; Makowska et al., 2011). Desensitization appears to target IFNα through the mediated interaction of the ISG-encoded ubiquitin-specific peptidase 18 (USP18) with the cognate receptor subunit IFNAR2 (Sarasin-Filipowicz et al., 2009; Francois-Newton et al., 2011). In contrast, IFNλ and IFNα were shown to be unaffected by repeated stimulation of the IFN transduction pathway (Makowska et al., 2011). Thus, even though both IFNα and IFNλ act through a common JAK-STAT pathway, they appear to be governed by differential regulatory control mechanisms. Further experiments will be required to address the impact of USP18 expression on the IFNα signaling pathway.

Previous reports have suggested that NSSA plays an important role in the regulation of IFN signaling by interacting directly with STAT1 and inhibiting its phosphorylation (Ian et al., 2007; Kumthip et al., 2012). Herein, resistance phenotype profiles were associated with reduced phosphorylation levels of both STAT1 and STAT2, once again underlying a general alteration in the host pathway of our replicon cell lines. Hence, this defect in IFN signaling may be a functional consequence of reduced expression of the upstream cellular mediator components of the JAK-STAT pathway. To support this assumption, it was found that IFNλ-resistant cell populations have reduced expression of the janus kinases Jak1 and Tyk2. In line with these observations, several studies have reported the establishment of IFNα-resistant replicon cell lines with a defect in the JAK-STAT signaling (Hazari et al., 2007; Zhu et al., 2005). In agreement with our results, Hazari et al. (2007) demonstrated that the resistant phenotype associated with IFNλ results from reduced expression of Jak1 and Tyk2, ultimately leading to defective phosphorylation of STAT proteins in replicon cells. It is therefore not surprising that IFNλ would follow a similar path of resistance development since both types of cytokines signal through the JAK-STAT pathway in order to induce their overlapping biological activities. Furthermore, our immunofluorescence analysis revealed an alteration in the subcellular distribution of the IFNAR1 chain and the IL-10Rβ chain in the IFN-resistant cell populations (Fig. 4). In the case of the type I IFN, a direct interplay between Tyk2 and the short-lived IFNAR1 protein has been shown to influence the localization and proteolysis fate of this co-receptor subunit, in turn regulating responses to IFNα (Ragimbeau et al., 2003; Marijanovic et al., 2006). Subsequently, functional disruption of the IFNAR1 was shown to play an important role in resistance to IFNα in replicon cells (Datta et al., 2011; Naka et al., 2005). Although the affinity of IFNAR1 for IFNα is weak, its tethering is crucial for the efficient assembly and functional stability of the ternary ligand-receptor complex. Current knowledge regarding the IL-10Rβ function in the IFNα receptor complex is limited to recruitment and binding to Tyk2. With other IL-10 related cytokines, downstream signaling events upon ligand stimulation are not dependent on IL-10Rβ but rather on their respective co-receptor chains (Donnelly et al., 2004; Sheikh et al., 2004). Unfortunately, the lack of reliable staining antibodies for the detection of the IL-28Rα co-receptor protein in conjunction with its putative low expression has hampered immunofluorescence studies of subcellular distribution. Therefore, we cannot rule out that the reduced expression of Jak1 observed
in the IFNα-resistant cell population may have a combined effect on the physiological expression of the IFNα receptor and ultimately on the downstream JAK-STAT signaling cascade. Unlike IL-10Rβ, the expression levels of IL-28Ra mRNA have been shown to vary greatly among tissues and, as a consequence, its expression is believed to be a limiting factor in the responsiveness to IFNλ stimulation (Witte et al., 2009). In contrast to the IFNAR2 chain which is highly expressed in many cell populations, levels of Tyk2 may set a lower sensitivity threshold of the more restrictive IL-28Ra chain by modulating the surface density of IL-10Rβ required for proper stability of the IFNλ receptor complex. Further studies will need to address the dynamics between the Janus kinases and the co-receptor subunits of IFNλ.

Although multiple models for the limited efficacy of alfa treatment against HCV infection have been proposed, its underlying mechanism(s) remains elusive. Ongoing clinical studies have indicated that Lambda may offer an alternative option for the treatment of chronic HCV. Regimens that include Lambda have resulted in more rapid declines in HCV RNA in patients when compared with alfa-containing regimens, leading to improved viral response rates at Weeks 4 and 12 of treatment in GT1 compared with alfa-containing regimens (Zeuzem et al., 2012; Muir et al., 2012). Therefore, clarification of non-responsiveness in these patient populations still requires additional work for future clinical guidance. Our in vitro data imply that functional cellular deficiencies in the JAK-STAT signaling pathway may provide a basic platform for understanding Lambda response rates in HCV patients. The implications of these in vitro results will be explored further to determine any correlation with in vivo findings.

Materials and methods

Cell lines and compounds

Human hepatoma Huh-7 cells that stably maintain the HCV subgenomic replicon representing genotype (GT) 1 subtype a (GT1a) (H77c strain: NCBI reference NC_004102.1) carrying an in-frame humanized renilla luciferase gene was previously described (McPhee et al., 2012b). Huh-7 cells that stably maintain a HCV GT1a subgenomic replicon with a specific amino acid substitution at NS3 protease residue R155 (NS3-R155K) was established following selection in the presence of G418 as previously described (McPhee et al., 2012b). The bovine viral diarrhea virus (BVDV) replicon genome carrying an in-frame luciferase gene was previously described (O’Boyle et al., 2005). The unpegylated recombinant human IFNa2b (rIFNa) was purchased from Myoderm Medical Supply (Norristown, PA). The unpegylated recombinant human IL-29 (rIFNλ) was synthesized by ZyMoGenetics, Inc (Seattle, WA). The structures and selective antiviral properties of the HCV NS3 protease inhibitor (NS3 PI) asunaprevir (AV; BMS-650032), and of the BVDV inhibitor, compound 1453, were previously described (McPhee et al., 2012a, 2012b; Sun et al., 2003).

Generation of IFN-resistant replicons

To generate stable IFN-resistant cell populations (IFNa2 or IFNλ), GT1a replicon cell lines were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% FBS, 2 mM l-glutamine, 0.5 mg/ml G418, containing various concentrations of rIFNa or rIFNλ, representing 30 or 100 times the 50% effective concentration (EC50) values determined (rIFNa EC50=50 IU/ml; rIFNλ EC50=2 ng/ml). Cells were passaged at 3–4 day intervals to maintain a sub-confluent monolayer for greater than 60 days, and IFNs were replenished in fresh media at the desired concentrations. Each replicon cell line propagated in parallel in the absence of IFN treatment and served as controls. The genotype and phenotype of the cell populations selected under IFN selective pressure were monitored to ensure continuous replication of the HCV replicon.

Phenotypic analysis

The susceptibility phenotype of the selected replicon cell populations to different classes of replication inhibitors was measured using either the intrinsic renilla luciferase activity of the stable HCV replicon, or the intrinsic firefly luciferase activity of a BVDV replicon transiently expressed in cells. For HCV phenotypic profiling, replicon cells were incubated for 3 days (37 °C, 5% CO2) in 96-well black-clear bottom plates (Costar 3614; BD Biosciences, San Diego, CA) in the presence of 3-fold serial dilutions of test compound in dimethyl sulfoxide (DMSO) or in Dulbecco’s phosphate buffered saline (D-PBS). HCV RNA replication was measured using a homogenous renilla luciferase substrate (EnduSure E6481; Promega, Madison, WI) according to manufacturer’s instructions. For transient phenotypic profiling, BVDV replicon in vitro RNA transcript was prepared from Sca I digested plasmid templates using the T7 RiboMax Express Large Scale Production System (P1320; Promega, Madison, WI) according to manufacturer’s instructions. Subsequently, 10 μg of BVDV RNA was electroporated into HCV replicon cell lines using a Bio-Rad GenePulser system (Bio-Rad Laboratories, Hercules, CA). Following electroporation, cells were diluted in DMEM supplemented with 10% FBS and seeded in 96-well black-clear bottom plates in the presence of 3-fold serial dilutions of test compound in DMSO or in D-PBS for 3 days. Replication of BVDV replicon in cells was measured using the firefly luciferase assay system (Promega, Madison, WI) according to manufacturer’s instructions. The 50% effective concentration (EC50) values were calculated with XLife software (version 2.0; IDBusiness Solutions, Burlington, MA) using a 4-parameter logistic equation: \[ y = A + \frac{B - A}{1 + \left(\frac{C}{x}\right)^D}. \]

Genotyping of IFN-resistant replicons

Total RNA was isolated from selected replicon cell populations using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA). First strand cDNA was synthesized from a specific GT1a primer with SuperScript II reverse transcriptase reagents (Invitrogen Corporation, Carlsbad, CA). PCR was then performed with Platinum Taq high-fidelity DNA polymerase (Invitrogen Corporation, Carlsbad, CA). The NS3 to NS5B coding region was amplified with genotype 1a primers; 5’–TGAATGTCGTGAAGGAACGCAG–3’ (forward) and 5’–GGAGCTTACCCCAACTT–3’ (reverse). Purified amplicons were subjected to sequence analysis and alignments were performed using Sequencher software (Gene Codes, Ann Arbor, MI).

IFN-signaling reporter assays

The IFN-mediated regulation of a pISRE-driven luciferase cistransporter plasmid (Agilent Technologies, Santa Clara, CA) that contains five direct repeats of the ISRE was monitored in the IFN-resistant replicon cell populations upon stimulation with rIFNa or rIFNλ. Briefly, cells seeded overnight in DMEM supplemented with 10% FBS were transfected with 0.25 μg of the pISRE-luciferase plasmid using FuGENE 6 transfection reagent (Roche Diagnostic Corporation, Indianapolis, IN). rIFNa or rIFNλ was added to the cultures at various concentrations, and cells were incubated for 24 h at 37 °C in 5% CO2. To measure luciferase activity, cells were washed with D-PBS, harvested with 1 times luciferase cell culture lysis buffer and incubated at room
temperature for 15 min with gentle agitation. Cell lysates were then transferred to 96-well solid white plates in triplicate (OptiPlate 6005290; Perkin-Elmer Life Sciences, Waltham, MA) and firefly luciferase substrate added to each well. Luminescence was measured immediately using a TopCount NXT counter.

**Western immunoblot analysis**

Cell lysates were prepared from replicon cells and the expression of various JAK-STAT signaling proteins was monitored by Western immunoblot analysis. Antibodies to Jak1, Tyk2, STAT1, phospho-STAT1 (Tyr701), phospho-STAT2 (Tyr690) and β-actin were purchased from Cell Signaling (Beverly, MA). Antibodies to IFNAR1, IFNAR2 and IL-28Rα were purchased from SABiosciences (Friedlander, MD). JAK-STAT signaling PCR array analysis

Replicon cells were seeded onto chamber slides (Nalge Nunc International, Denmark) at a density of 5 × 10⁵ cells per well overnight then fixed with 4% paraformaldehyde and permealized prior to antibody staining. Cells were incubated overnight at room temperature with antibodies to IFNAR1 (Lifespan Biosciences, Seattle, WA), IL-10Rβ (R&D Systems, Minneapolis, MN) or to Tyk2 (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was incubated with the indicated primary antibody and subsequently with the appropriate secondary antibody conjugated with horseradish peroxidase. Proteins were detected using the Amersham ECL Plus Western blotting detection system (GE Healthcare, Buckinghamshire, UK) and chemiluminescent signals were visualized using Kodak BioMax film (Carestream Health, Rochester, NY). Relative protein levels detected were quantified using Fujifilm MultiGauge software and the arbitrary unit (AU) reported from the average of three independent analyses.

**Fluorescence microscopy analysis**

Replicon cells were seeded onto chamber slides (Nalge Nunc International, Denmark) at a density of 5 × 10⁵ cells per well overnight then fixed with 4% paraformaldehyde and permealized prior to antibody staining. Cells were incubated overnight at room temperature with antibodies to IFNAR1 (Lifespan Biosciences, Seattle, WA), IL-10Rβ (R&D Systems, Minneapolis, MN) or to Tyk2 (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were subsequently incubated for 1 h at room temperature with Alexa Fluor® 488- and Alexa Fluor® 594-coupled secondary antibodies, respectively, as recommended by the manufacturer’s instructions (Molecular Probes, Grand Island, NY). Nuclei were counterstained with Hoechst dye and cells were visualized under a Nikon Eclipse TE 300 microscope.

**JAK-STAT signaling PCR array analysis**

Replicon cells seeded overnight in 6-well assay plates (Becton Dickinson, Franklin Lakes, NJ) at a density of 1.5 × 10⁶ cells per well were treated with rIFNα or rIFNγ. 10 or 100 ng/ml for 24 h at 37 °C in 5% CO₂. IFN-treated and untreated replicon cells were then washed once with D-PBS and lysed with 100 μL of RIPA buffer (SIGMA) supplemented with protease inhibitors and phosphatase inhibitors cocktails (SIGMA). Equal amounts of protein lysates were separated by NuPAGE 3-8% gel and then transferred onto an InvitroCell PVDF membrane (Innogen, Carlsbad, CA). The membrane was incubated with the indicated primary antibody and subsequently with the appropriate secondary antibody conjugated with horseradish peroxidase. Proteins were detected using the Amer sham ECL Plus Western blotting detection system (GE healthcare, Buckinghamshire, UK) and chemiluminescent signals were visualized using Kodak BioMax film (Carestream Health, Rochester, NY). Relative protein levels detected were quantified using Fujifilm MultiGauge software and the arbitrary unit (AU) reported from the average of three independent analyses.


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