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Lambda and alpha interferons inhibit hepatitis B virus replication through a common molecular mechanism but with different *in vivo* activities

Nicole E. Pagliaccetti, Esther N. Chu, Christopher R. Bolen, Steven H. Kleinstein, Michael D. Robek *

Department of Pathology, Yale University School of Medicine, New Haven, CT, USA

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ABSTRACT

The type III interferons (IFN-λ1, 2, and 3) induce an antiviral response similar to IFN-α/β, but mediate their activity through a unique receptor. We found that like IFN-α/β, IFN-λ prevents the assembly of HBV capsids, demonstrating convergence of the two signaling pathways through a single antiviral mechanism. In contrast to IFN-λ, the structurally related cytokine interleukin (IL)-22 only minimally reduced HBV replication. The transcriptional program activated by IL-22 displayed little similarity to that induced by IFN-λ, but instead resembled the response elicited by IL-6. We also found that murine IFN-λ2 had only weak antiviral activity against HBV in the liver of transgenic mice, and that human IFN-λ2 activity in serum correlated with the sensitivity of the cytokine to proteases. These results demonstrate that the IFN-α/β and IFN-λ anti-HBV responses operate through a single molecular mechanism, and support the notion that IFN-λ plays a local, rather than systemic, role in antiviral immunity.

Introduction

Hepatitis B virus (HBV) is a hepatotropic virus that causes either acute or chronic hepatitis in infected individuals. Currently, more than 300 million people worldwide are chronically infected with HBV and are at risk for developing hepatocellular carcinoma (Liang, 2009). It is well established that interferon (IFN)-γ production is important for the non-cytopathic control of HBV replication (Guidotti and Chisari, 2006). IFN-γ is secreted by cytotoxic T-lymphocytes and inhibits both HBV gene expression and replication (Guidotti et al., 1994, 1996). In contrast to IFN-γ, HBV does not induce IFN-α/β expression (Wieland and Chisari, 2005), thus enabling HBV to establish infection before the adaptive immune response can be launched. Although HBV does not activate IFN-α/β, the virus is sensitive to its antiviral effects. IFN-α has been used clinically to treat chronic HBV infection, but this treatment is only effective in a moderate proportion of patients (approximately 40%) and is associated with significant side effects (Perrillo, 2009), thus limiting its utility.

IFN-λ1, 2, and 3 are members of the class II α-helical cytokine family (Kotenko et al., 2003; Sheppard et al., 2003), which also includes IFN-α/β, IFN-γ, and the IL-10 family cytokines. Also known as the type III IFNs, these cytokines have functional properties similar to those of IFN-α/β, including the activation of Jak/STAT signaling and induction of IFN-stimulated response element-dependent genes. Furthermore, the IFN-λ response inhibits the replication of viruses such as vesicular stomatitis virus (VSV), encephalomyocarditis virus, and herpes simplex virus type 2 (HSV-2) (Ank et al., 2006; Kotenko et al., 2003; Sheppard et al., 2003). IFN-λ also blocks HBV and hepatitis C virus (HCV) replication in cell culture (Robek et al., 2005), but the molecular mechanism of this inhibition is unknown.

Despite their functional similarities to IFN-α/β, the type III IFNs structurally resemble the IL-10 family members, in particular IL-22 (Gad et al., 2009). The IFN-λ receptor consists of two subunits, one that is unique to the IFN-λs (IL-28R1), and the second is the IL-10 receptor subunit IL-10R2 (Kotenko et al., 2003; Sheppard et al., 2003), which is also a component of the IL-22 receptor (Kotenko et al., 2001; Xie et al., 2000). While IFN-λ performs antiviral functions, IL-22 increases microbial defense and protects certain types of tissues (including epithelia and liver) from damage (Radaeva et al., 2004; Wolk et al., 2004, 2006; Zenewicz et al., 2007). Although the IL-22 receptor is expressed on hepatocytes, recent evidence indicates that IL-22 lacks direct antiviral activity against HCV (Dambacher et al., 2008). However, IL-22 may offer protection to infected hepatocytes through other mechanisms, as mice lacking IL-22 are highly susceptible to hepatic damage due to inflammation (Zenewicz et al., 2007). It is also currently unknown if IL-22 influences the signaling or activity of IFN-λ, or if any crosstalk between receptors occurs.

Though the signaling and *in vitro* antiviral activity of IFN-λ has been studied in detail, much less is known about *in vivo* activity. The distribution of the IFN-λR complex differs between organs and tissue

* Corresponding author. Department of Pathology, Yale University School of Medicine, P.O. Box 208023, 310 Cedar Street LH315A, New Haven, CT 06520-8023, USA. Fax: +1 203 785 6127.
E-mail address: michael.robek@yale.edu (M.D. Robek).

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types (Ank et al., 2008; Kotenko et al., 2003; Sheppard et al., 2003; Sommereyns et al., 2008). Current evidence indicates that in mice, IFN-λ may systemically inhibit some viruses, but primarily acts locally to prevent infection (Ank et al., 2006; Bartlett et al., 2005; Mordstein et al., 2008). The IFN-λ response was found to be highest in epithelial cells and lower in cells of endothelial origin (Sommereyns et al., 2008). The tissue specificity of this response suggests that IFN-λ may have evolved to defend against viral infections in epithelial tissues (Sommereyns et al., 2008). It is unclear whether IFN-λ is active in the liver, as IFN-λ is expressed in the mouse liver following infection (Sommereyns et al., 2008), but was not found to protect mice from hepatotropic viruses when injected intraperitoneally (Mordstein et al., 2008).

We previously reported that HBV replication is blocked by IFN-λ (Robek et al., 2005), but a number of important questions remained regarding this finding: What is the molecular mechanism of this inhibition, and is this mechanism the same as that activated by IFN-α and IFN-γ? How does the antiviral activity of IFN-λ compare to other related cytokines such as IL-22? What regulatory mechanisms contribute to the *in vivo* activity of this cytokine? In this study, we found that IFN-λ inhibits HBV replication by limiting the formation of HBV RNA-containing capsids, identical to the molecular mechanism induced by IFN-α. Compared to IFN-λ, IL-22 displayed limited antiviral activity and induced a more restricted hepatocellular gene expression profile. We also found that IFN-λ2 only weakly inhibited virus replication in the liver of HBV transgenic mice after intravenous injection, and that human IFN-λ2 activity in serum correlated with the sensitivity of the cytokine to proteases. These studies further elucidate the molecular mechanisms of IFN-λ antiviral activity to a medically important virus.

Results

IFN-λ inhibits HBV replication by preventing assembly of HBV RNA-containing capsids

During the HBV replication cycle, viral RNA transcribed from nuclear covalently closed circular viral DNA is encapsidated by the viral core protein in the cytoplasm. After encapsidation, this pregenomic RNA is reverse-transcribed by the viral polymerase protein first to single-stranded and then to partially double-stranded relaxed circular DNA forms prior to virion release from the cell (Ganem and Prince, 2004). It has previously been shown that IFN-α/β and IFN-γ inhibit HBV replication by preventing the assembly of HBV RNA-containing capsids (Wieland et al., 2005). It was therefore possible that IFN-λ either inhibits a similar step in replication, or a different point in the HBV life cycle. To determine the step in the HBV replication cycle where IFN-λ acts, we used an immortalized murine hepatocyte cell line (TREHBV-V) that expresses HBV under a tetracycline-inducible promoter (Wieland et al., 2005). The HBV genome in these cells contains a mutation in the polymerase catalytic domain (YMDD), preventing the reverse transcription of viral RNA into DNA. Therefore, immature RNA-containing capsids accumulate within the cytoplasm without being converted into DNA and released from the cell.

We first determined if IFN-λ treatment reduced the levels of encapsidated HBV RNA in the cell. For these studies, we used both human and murine IFN-λ, as we had previously determined that the murine cells are capable of responding to the human cytokine (data not shown). Cells were treated with 600 pg/ml of IFN-α(A/D), 100 pg/ml of human IFN-λ1, or 100 pg/ml murine IFN-λ2 for six hours prior to induction with doxycycline (Fig. 1A). These concentrations were utilized because they represent the minimal amount of each cytokine necessary to reproducibly inhibit HBV replication in immortalized mouse hepatocytes (data not shown). Four days post-induction, total and encapsidated RNA were harvested and analyzed by Northern blot. Similar to IFN-α, neither human IFN-λ1 nor murine IFN-λ2 significantly reduced the total amount of HBV mRNA in the cells (Fig. 1B). However, treatment with either IFN-λ subtype resulted in a similar complete loss of encapsidated RNA (Fig. 1B), as had previously been found with IFN-α/β and IFN-γ (Wieland et al., 2005).

The loss of encapsidated RNA induced by IFN-λ could be due to either an increase in the degradation rate of preformed capsids, or the prevention of HBV RNA-containing capsid formation. To determine if IFN-λ promotes capsid degradation, cells were induced to express HBV RNA for four days, then incubated for 84 h in the absence of doxycycline to turn off new RNA synthesis and allow the previously expressed RNA to become encapsidated (Fig. 1A). The cells were then treated for 6, 12, 24, or 36 h with 100 pg/ml of human IFN-λ1, and encapsidated RNA was compared to untreated controls over the 36-hour time course. Encapsidated RNA in IFN-λ-treated cells disappeared at the same rate as in untreated control cells (Figs. 1C and D), indicating that IFN-λ does not promote the degradation of HBV RNA-containing capsids. From these data we conclude that like IFN-α/β and IFN-γ, the cellular antiviral response elicited by IFN-λ inhibits HBV replication by preventing the assembly of HBV capsids in the cytoplasm.

Neither IFN-λ nor IFN-α inhibits HBV in HepG2-derived cells

We next examined the anti-HBV activity of IFN-α and IFN-λ using a human hepatoma derived cell line (HepG2) encoding the HBV genome [HepG2(2.2.15)] (Sells et al., 1987). Unlike the immortalized hepatocytes, HepG2 cells have inherent defects in the IFN-mediated antiviral response (Keskinen et al., 1999). In fact, IFN-α/β only modestly inhibits HBV replication in HepG2 cells (Biermer et al., 2003). Both STAT-1 and STAT-2 were phosphorylated to similar extents 15 min after treatment with either 600 pg/ml IFN-α or 100 ng/ml IFN-λ in HepG2(2.2.15) cells, indicating that the cytokine receptors are present and responsive in this cell line (Fig. 2A). However, following a 24-hour treatment with the cytokines, neither IFN-α nor IFN-λ induced a significant reduction in either HBV DNA or RNA (Fig. 2B). As it is well established that IFN-α inhibits HBV replication in other systems (McClary et al., 2000; Pasquetto et al., 2002; Wieland et al., 2000, 2003, 2005), these data suggest that there is an inherent defect in the IFN response in HepG2 cells that prevents a full antiviral response against HBV. Therefore, like IFN-α, IFN-λ inhibits HBV replication in cells that can fully respond to the antiviral signals induced by the cytokines, but does not in cell lines such as HepG2 that cannot.

Combinations of IFN-λ with IFN-α/β or IFN-γ induce only modest increases in antiviral activity against HBV

We previously demonstrated that combinations of IFN-λ and IFN-α or IFN-γ inhibit HCV and VSV replication to a greater extent than the individual cytokine treatments alone (Pagliaccetti et al., 2008). The increased effects were additive when IFN-λ was combined with IFN-α and synergistic with the combination of IFN-λ and IFN-γ. To determine if these combined antiviral effects were also observed in the inhibition of HBV, TREHBV cells were treated in triplicate with 1.2 ng/ml of murine IFN-β, 10 ng/ml of murine IFN-γ, or 100 pg/ml of murine IFN-λ2 alone or in combination for 12, 24, or 48 h. These concentrations were chosen based upon the fact that they induce similar levels of HBV DNA reduction (60–80%) at 24 h post-treatment when used alone. Cytoplasmic DNA was harvested from the cells and HBV replication forms were analyzed by Southern blot (Fig. 3). Although we observed greater HBV inhibition with the combined treatments than with a single cytokine alone, the increases were no greater than an additive (i.e. dose-dependent) effect, consistent with the convergence of the IFN-stimulated antiviral pathways through a single downstream antiviral mechanism.

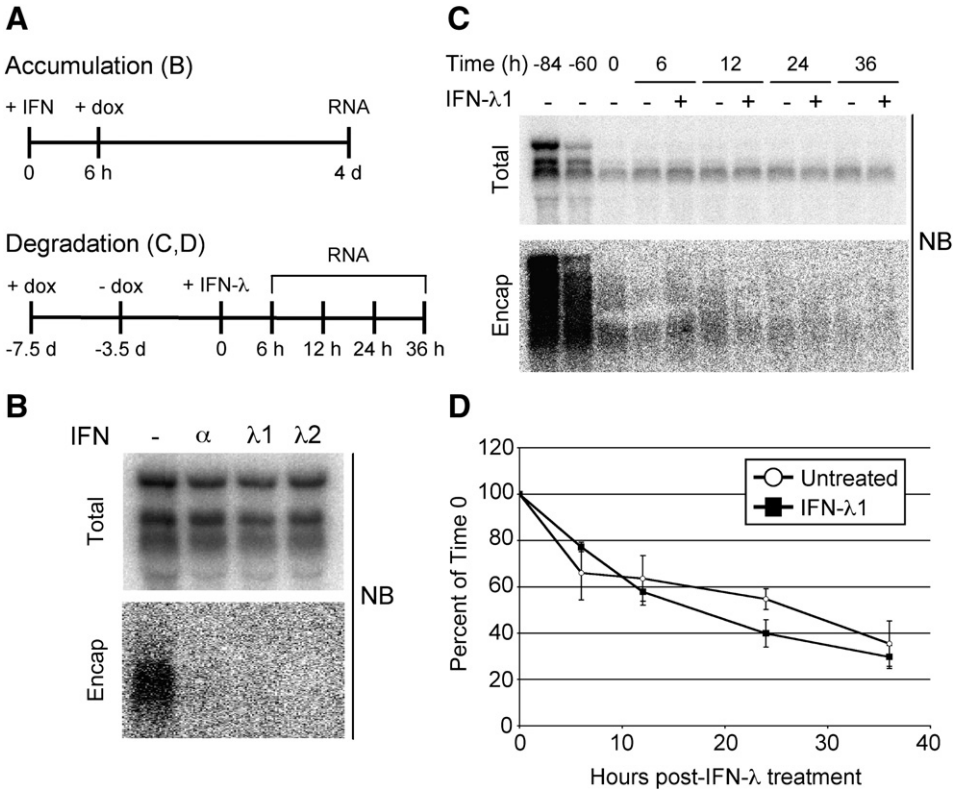

Fig. 1. IFN-λ inhibits HBV replication by preventing the assembly of RNA-containing capsids. (A) Schematic of experimental protocols for determining inhibition of capsid accumulation or degradation. (B) TREHBV-V cells were treated with 600 pg/ml of IFN-α(A/D), 100 pg/ml of human IFN-λ1, or 100 pg/ml of mouse IFN-λ2 for six hours prior to treatment with 1 μg/ml doxycycline (dox) to induce HBV RNA expression. Four days after induction, total and encapsidated RNA were prepared and analyzed by Northern blot. Blot shown is representative of three experiments. (C) TREHBV-V cells were induced to express HBV RNA with 1 μg/ml doxycycline for four days. Cells were then cultured in media without doxycycline for 86 h before treatment with 100 pg/ml of IFN-λ for 6, 12, 24 or 36 h. Total or encapsidated HBV RNA was measured by Northern blot. Blot shown is representative of three experiments. (D) Quantification of encapsidated HBV RNA as determined from phosphorimager analysis. Data points represent the mean of three replicates and error bars indicate standard error of mean.

Comparison of IFN-λ and IL-22 inhibition of HBV replication

IFN-λ and IL-22 are both members of the class II α helical cytokine family and share a receptor subunit (IL-10R2) (Donnelly et al., 2004), and a recent study has demonstrated that these cytokines are also structurally similar (Gad et al., 2009). These similarities raised the possibility that IL-22 may also have direct antiviral activity against HBV, or may influence the IFN-λ-mediated inhibition of the virus. HBV-Met cells were treated with 100 ng/ml of murine IL-22 for 1, 3, or 5 days, and HBV DNA and RNA levels were measured by Southern blot or RT-qPCR, respectively (Figs. 4A and B). IL-22 treatment reduced HBV DNA levels (Fig. 4A), but this inhibition was much weaker (~2-fold reduction) compared to the strong inhibition by IFN-λ (>10-fold reduction, as shown in Fig. 3). Although modest, this inhibition was reproducible and was also found in the Tet-responsive cell line TREHBV (data not shown), thus ruling out promoter-specific effects. In addition, IL-22 had no consistent effect on HBV RNA levels (Fig. 4B), nor did it influence the antiviral activity of IFN-λ (Fig. 4C). Therefore, unlike IFN-λ, the structurally related cytokine IL-22 has only limited antiviral activity against HBV.

Differences in hepatocellular response to IFN-λ and IL-22

To understand the differences in the IFN-λ and IL-22 responses to HBV, we examined the gene expression changes induced by these cytokines in immortalized mouse hepatocytes, and compared these changes to those induced by IFN-γ and IL-6. We chose these cytokines for comparison for two reasons. First, like IFN-α/β, IFN-γ potently inhibits HBV replication in hepatocytes (Guidotti et al., 1994, 1996), and the IL-22 gene occupies a position in the genome in close proximity to the IFN-γ gene (Dumoutier et al., 2000), raising the possibility that the two cytokines could possibly have some similar characteristics. Second, IL-22 induces a response in keratinocytes that displays some similarities to the acute phase response elicited by IL-6 (Wolk et al., 2006), but to our knowledge, these responses have not been directly compared side-by-side in liver cells.

We first confirmed that the c-Met immortalized mouse hepatocytes (MMHD3) respond to the cytokines. Consistent with reports that utilized other cell types (Andoh et al., 2005; Wolk et al., 2004, 2006), IFN-γ and IFN-λ2 primarily activated STAT-1 phosphorylation in the MMHD3 cells, while IL-6 and IL-22 predominately induced STAT-3 activation (Fig. 5A). We then performed a microarray gene expression analysis to compare the overall changes in gene expression induced by IFN-λ and IL-22. Hierarchical clustering of the gene expression data demonstrated that the IL-22 response is most similar to IL-6 (Fig. 5B). Many of the gene expression changes induced by IL-22 are mirrored in the IL-6 response, and several of these genes (including IFIT2, RSAD2, and USP18) move in the opposite direction following treatment with IFN-λ or IFN-γ. In order to ensure that the commonality between IL-22 and IL-6 went beyond their shared activation levels, we also performed a Gene Set Enrichment Analysis (GSEA) to identify functional groups that were enriched among the most differentially expressed genes. Since GSEA operates on a rank-ordered list of genes without taking into account the magnitude of differential expression, this method should be less sensitive to the overall lower levels of gene expression in the IL-22 and IL-6 responses. Although most of the gene sets that are significantly enriched ($q<0.1$) in the IL-22 response are also enriched in the other responses, the extensive overlap between the top-20 gene sets of IL-22 and IL-6, none of which are shared by IFN-λ or IFN-γ, confirms the similarity

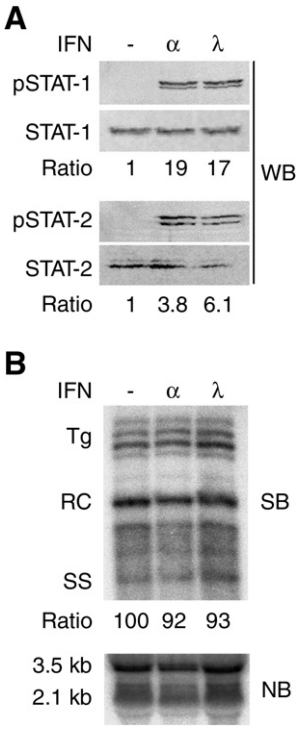

Fig. 2. Neither IFN-α nor IFN-λ inhibits HBV replication in HepG2 (2.2.15) cells. (A) Western blot (WB) analysis of phosphorylated and total STAT-1 and -2 in HepG2 (2.2.15) cells treated with 600 pg/ml of IFN-α(A/D) or 100 ng/ml of IFN-λ for 15 min. (B) HBV DNA and RNA replication forms in HepG2 (2.2.15) cells treated with 600 pg/ml of IFN-α(A/D) or 100 ng/ml of IFN-λ for 24 h. HBV relaxed circle (RC) and single-stranded DNA replication forms were measured by Southern blot (SB) analysis and compared to the integrated transgene (Tg). The HBV 3.5 and 2.1 kb RNAs were measured by Northern blot (NB). Southern and Northern blots shown are representative of two experiments.

between IL-22 and IL-6 identified by the hierarchical clustering (Fig. 5C).

Inhibition of HBV replication by IFN-λ in mice is weak compared to IFN-β or IFN-γ

Although IFN-α/β and IFN-λ both inhibit HBV replication in immortalized hepatocytes with similar efficiencies, the activity of these cytokines may differ in the liver *in vivo*. Therefore, we compared the antiviral activity of murine IFN-λ2 to that of IFN-β and IFN-γ in HBV transgenic mice. Animals were injected intravenously with the cytokines, and virus replication in the liver was measured 24 h later by Southern hybridization of HBV DNA. The inhibition of HBV replication was only modest in mice that received 100 μg IFN-λ (<50% reduction), as compared to mice that received 10 μg of either IFN-β or IFN-γ (>80% reduction) (Fig. 6). Therefore, compared to IFN-β or IFN-γ, mouse IFN-λ displays lower activity in the liver *in vivo* after i.v. injection.

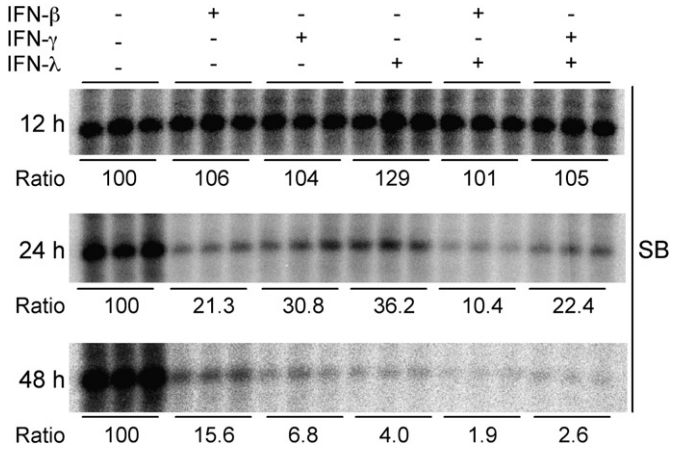

Fig. 3. Combinations of IFN-λ with IFN-α/β or IFN-γ induce greater antiviral activity than individual cytokines alone. TREHBV cells induced with 1 μg/ml Dox were treated in triplicate with 1.2 ng/ml of IFN-β, 10 ng/ml of IFN-γ, or 100 pg/ml of IFN-λ2 alone or in combination for 12, 24 or 48 h. Cytoplasmic HBV single-stranded DNA was measured by Southern blot (SB) analysis. Relaxed circle DNA forms displayed a similar pattern (data not shown). Ratios were determined by quantification of phosphorimager files.

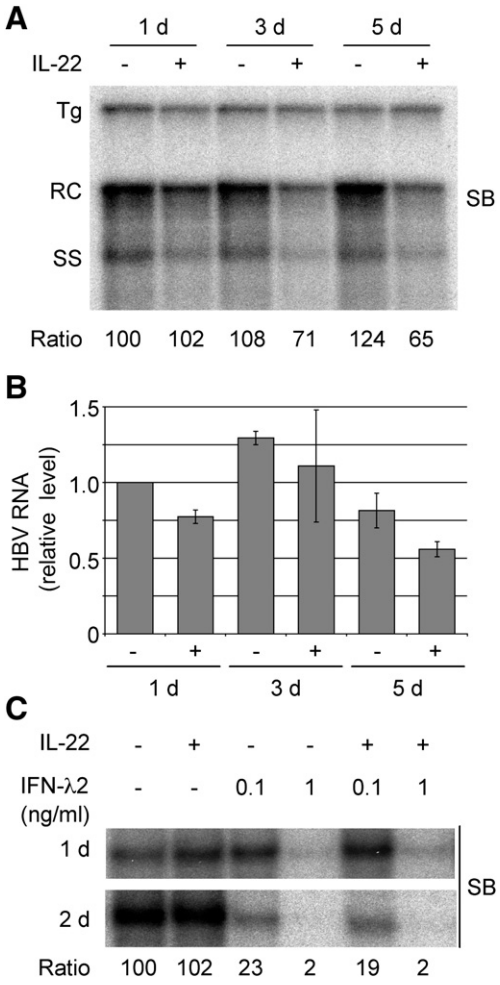

Fig. 4. IL-22 has only limited antiviral activity against HBV compared to IFN-λ. Differentiated HBV-Met cells that were treated with 100 ng/ml of IL-22 for 1, 3, or 5 days. HBV replication was measured by (A) Southern blot analysis of the HBV relaxed circle (RC) and single strand (SS) DNA replication forms or (B) Northern blot analysis of HBV RNA. (C) TREHBV cells were treated with 10 ng/ml of IL-22 or the indicated amounts of IFN-λ2 alone or in combination. The HBV single strand DNA replication form was measured by Southern blot 1 or 2 days post-treatment. Ratios were determined by quantification of phosphorimager files.

Sensitivity of IFN-λ2 to a serum protease

The relatively low activity of mouse IFN-λ2 in the liver after i.v. injection led us to question whether the human type III IFNs might be sensitive to inactivation by serum proteins. We therefore performed an *in vitro* assay to measure human IFN-λ1 and IFN-λ2 activity over time in pooled normal human serum. One microgram of IFN-λ1 or IFN-λ2, or 50 ng of IFN-α2a was incubated in 100 μl serum for 3, 9, or 24 h at 37 °C, and cytokine activity was then determined by treating Huh7 cells with aliquots (final concentration 10 ng/ml for IFN-λ or 50 pg/ml IFN-α2a) for 24 h and measuring expression of a

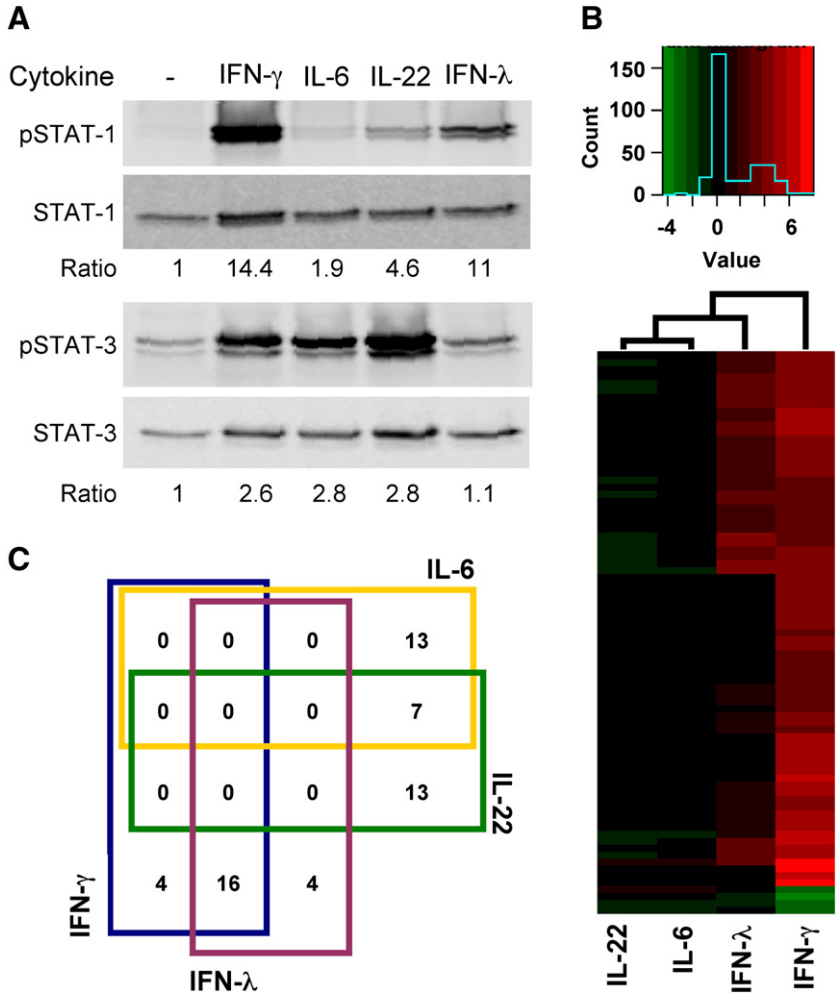

Fig. 5. Differential STAT phosphorylation and transcriptional programs predict anti-HBV activity of IFN-λ and IL-22. (A) Phosphorylated and total STAT-1 and STAT-3 expression in MMHD3 cells 15 min after stimulation with 100 ng/ml IL-22, IL-6, IFN-λ, or IFN-γ. (B) Heatmap of differentially expressed genes. Colors indicate the log2 average fold change of genes with an absolute fold change greater than eight in at least one of the treatment groups. The rows and columns are clustered using hierarchical clustering. A similar pattern of expression is observed when using lower differential expression cutoffs (data not shown). (C) Functional similarity between IL-22 and IL-6. GSEA was used to determine the top-20 enriched functional groups for each of the treatments. Numbers indicate the extent of overlap between up-regulated gene sets across the different treatments (rectangles). The list of genes displayed in (B) and the functional groups shown in (C) are provided in the Supplementary data.

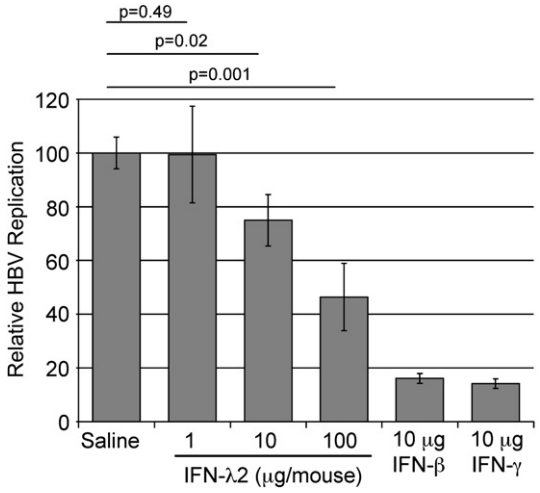

Fig. 6. Modest anti-HBV activity of IFN-λ in mice. HBV transgenic mice (3–6 animals per group) were injected intravenously with saline or the indicated amounts of murine IFN-λ2, IFN-β, or IFN-γ. HBV replication in the liver was measured 24 h post-injection by Southern blot analysis of HBV relaxed circle and single strand DNA replication forms. Data represent mean total HBV DNA quantified by phosphorimager analysis, and error bars indicate standard error of the mean. *P* values indicate result of Student's *t*-test.

representative IFN-stimulated gene (MxA) by quantitative real-time RT-PCR. These final concentrations were used because they are the minimal amounts necessary to induce maximal MxA expression for each cytokine (approximately 2000-, 1000-, and 500-fold MxA induction for IFN-α2a, IFN-λ1, and IFN-λ2, respectively; data not shown). As shown in Fig. 7A, IFN-α2a maintained activity throughout the time course, dropping only about 15% over 24 h. Similarly, IFN-λ1 displayed only a relatively moderate loss of activity (60% reduction after 24 h). However, IFN-λ2 showed the greatest loss of activity, decreasing by 40% after only 3 h and 98% after 24 h.

We hypothesized that the significant loss in IFN-λ2 activity may be due to degradation by proteases in the serum. Therefore, we incubated IFN-λ2 in the presence or absence of a protease inhibitor cocktail for 24 h as described above. The addition of the inhibitor cocktail to the serum prevented the loss of IFN-λ2 activity for the full 24 h (Fig. 7B). We also examined the levels of the IFN proteins in the serum over time. While the amount of IFN-α and IFN-λ1 stayed relatively constant over the 24-hour time period, the level of full-length IFN-λ2 rapidly decreased, and was replaced by a product of smaller molecular weight (Fig. 7C). Furthermore, the protease inhibitor cocktail blocked this cleavage/degradation (Fig. 7C). We then tested the inhibitors comprising the cocktail individually, and while leupeptin, aprotinin, bestatin, E64, and EDTA had no effect on IFN-λ2 cleavage (data not shown), the irreversible serine protease inhibitor AEBSF blocked

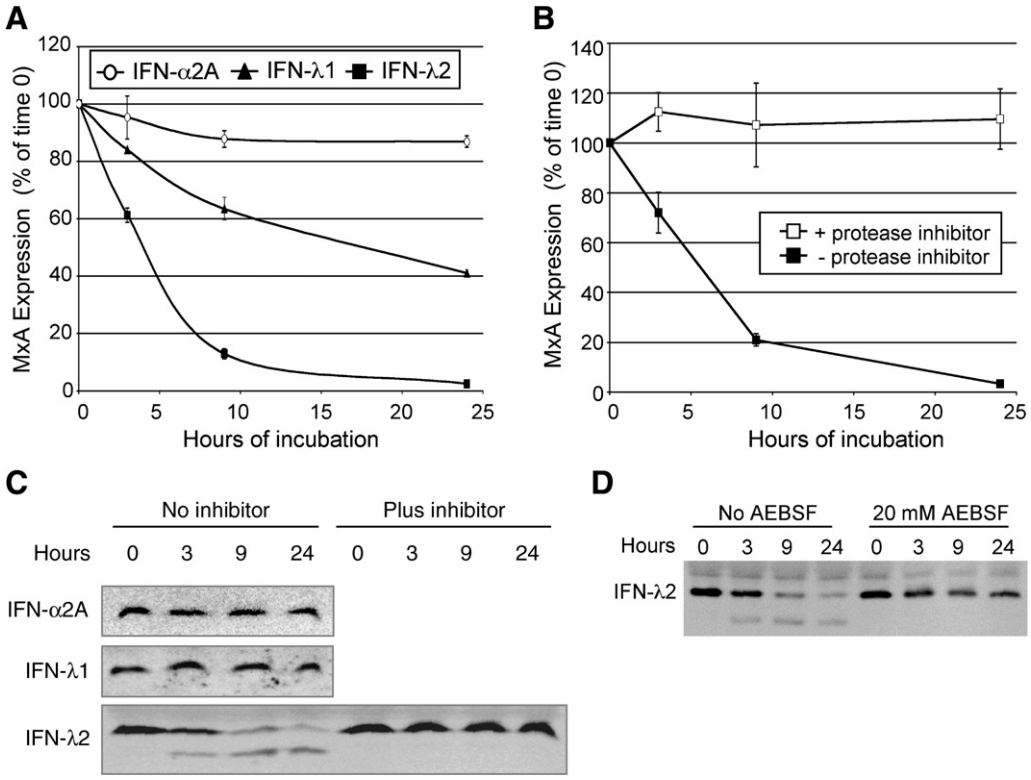

Fig. 7. Protease inhibitors prevent loss of activity and degradation of IFN-λ in serum. (A) Activity of human IFN-α2a, IFN-λ1, and IFN-λ2 following incubation in human serum at 37 °C for 3, 9, or 24 h. Quantification of activity was performed by treating Huh-7 cells with cytokine/serum for 24 h, followed by quantitative RT-PCR analysis of the representative IFN-stimulated gene MxA. Data are expressed as MxA expression relative to time 0 and are normalized to GAPDH expression. (B) IFN-λ2 was incubated in human serum at 37 °C for 3, 9, or 24 h in the absence or presence of a protease inhibitor cocktail. Quantification of activity was performed by treating Huh-7 cells with cytokine/serum for 24 h, followed by quantitative RT-PCR analysis of the representative IFN-stimulated gene MxA. Data points are the average of three experiments and error bars indicate SEM. (C) Western blots of IFN-α2a, IFN-λ1, and IFN-λ2 at the indicated time points. (D) AEBSF prevents IFN-λ2 cleavage in serum.

cleavage (Fig. 7D). Taken together, these results suggest that human IFN-λ2 may display lower activity compared to other IFNs due to its susceptibility to a serum serine protease.

Thrombin efficiently cleaves human IFN-λ2

Amino acid sequence alignment and analysis of potential protease cleavage sites (http://www.expasy.ch/tools/peptidecutter/) identified a consensus thrombin cleavage site present in human IFN-λ2 but absent in IFN-λ1 (Fig. 8A). Consistent with this finding, we found that recombinant human thrombin efficiently cleaved human IFN-λ2 in a manner similar to that observed with human serum (Fig. 8B). Similarly, mouse thrombin cleaved mouse IFN-λ2, although the size of the product differed from that observed with the human cytokine, which may reflect either a different cleavage site or different mobility of the product in SDS-PAGE. In contrast to IFN-λ2, IFN-λ1 was only cleaved at the highest thrombin concentration (0.1 NIH units) and for the longest incubation periods (2–4 h) (Fig. 8B). To determine if thrombin was necessary for IFN-λ2 cleavage in serum, we also tested the effect of the thrombin-specific inhibitor hirudin on IFN-λ degradation (Fig. 8C). The addition of ≥5 antithrombin units (ATU) per 50 μl serum completely protected 1 μg IFN-λ2 as well as the more resistant IFN-λ1 from cleavage during 24 h of incubation. Therefore, compared to IFN-λ1, IFN-λ2 displays greater sensitivity to thrombin proteolytic activity.

Discussion

Molecular mechanism of HBV inhibition

We previously demonstrated that type III interferon inhibits HBV replication in immortalized mouse hepatocytes (Robek et al., 2005). Although IFN-α and IFN-λ signal through distinct receptors, they induce nearly identical patterns of gene expression (Doyle et al., 2006; Marcello et al., 2006; Pagliaccetti et al., 2008; Zhou et al., 2007). Here, we show that like IFN-α/β and IFN-γ, the antiviral response induced by IFN-λ inhibits HBV replication by preventing the formation of HBV RNA-containing capsids in the cytoplasm, demonstrating that the three innate antiviral pathways are functionally redundant with respect to HBV inhibition. However, the IFN-induced protein that actually inhibits HBV replication has not been identified, and remains an important area for future investigation.

The effect of IFN-λ on HBV replication in cells derived from the human hepatoma cell line HepG2 (WT-10; PEB8) has been previously described (Doyle et al., 2006; Hong et al., 2007). In contrast to our previous results using immortalized mouse hepatocytes, these studies found relatively weak antiviral activity of IFN-λ against HBV replication in HepG2-derived cells (≤30% reduction with ≥1000 ng/ml IFN-λ1 in Hong et al.; ≈60% reduction with ≥3 ng/ml PEG-IFN-λ1 in Doyle et al.), a finding which we also confirm here. However, unlike the immortalized hepatocytes used in our studies, HepG2 cells are known to have inherent defects in the IFN-mediated antiviral response (Keskinen et al., 1999). Despite the fact that they respond to IFN-α/β as measured by increased STAT-1 phosphorylation and IFN-stimulated gene expression (Melen et al., 2000), the replication of viruses such as influenza and VSV is only weakly inhibited in these cells (Keskinen et al., 1999). In fact, HBV replication is known to be much more sensitive to TNF-α than to IFN-α/β in HepG2 cells (Biermer et al., 2003), even though IFN-α/β inhibitory activity against HBV is well-characterized in other cell culture and *in vivo* models (McClary et al., 2000; Pasquetto et al., 2002; Wieland et al., 2000, 2003, 2005). Therefore, like IFN-α, IFN-λ inhibits HBV replication in hepatocyte-derived cells that can fully respond to the

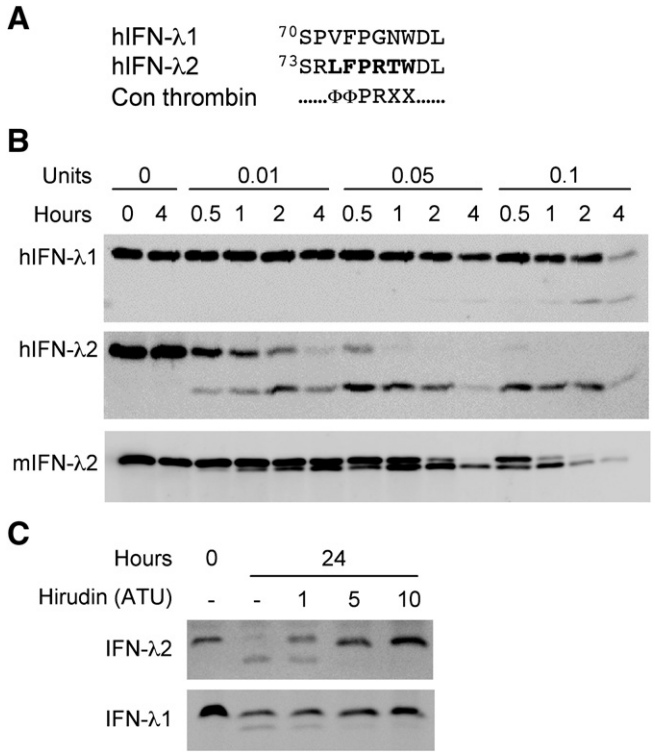

Fig. 8. IFN-λ2 is sensitive to cleavage by thrombin. (A) Amino acid sequence alignment of IFN-λ1 and IFN-λ2 in the region of a consensus thrombin cleavage site (ΦΦPRXX; where Φ is a hydrophobic amino acid and X is any non-acidic amino acid). (B) Human IFN-λ1 and IFN-λ2 or mouse IFN-λ2 were incubated in the presence of the indicated NIH units of thrombin (human or mouse, respectively) in cleavage buffer (50 mM Tris–HCl pH 8.0, 2.5 mM $CaCl_2$, 150 mM NaCl) in a total volume of 30 μl. At the time points indicated, 5 μl aliquots were removed and combined with 5 μl 2× SDS sample buffer for Western blot analysis. (C) One microgram of human IFN-λ2 or IFN-λ1 was incubated in 50 μl serum in the presence of 1, 5, or 10 ATU of hirudin for 24 h, and cleavage was determined by Western blot.

antiviral signals induced by the cytokines, but this effect is weaker in cell lines such as HepG2 that have defects in this pathway.

IL-22 and the innate anti-HBV response

Gad et al. recently determined the crystal structure of IFN-λ and found a close similarity to the structure of the IL-10 family cytokine IL-22 (Gad et al., 2009). Because IFN-λ has antiviral activity and IL-22 appears to possess other antimicrobial activities, it was suggested that these cytokines may both act to defend epithelial cells from different types of infection (Gad et al., 2009). Though recent evidence indicates that IL-22 does not protect cells from HCV infection (Dambacher et al., 2008), it may provide protection through other mechanisms as IL-22 has been identified as a survival factor for hepatocytes, and IL-22 knockout mice are more susceptible to inflammatory hepatic damage (Radaeva et al., 2004; Zenewicz et al., 2007). To further characterize the activity of IL-22 in hepatocytes, we tested its antiviral effects against HBV both alone and in combination with IFN-λ. Although IL-22 was able to reduce HBV replication, compared to IFN-λ the response was much weaker (2-fold inhibition compared to 20-fold) and required both higher concentrations (100 ng IL-22/ml vs. 100 pg IFN-λ/ml) and a longer treatment time (3–5 days vs. 1–2 days). Thus, unlike IFN-λ, which strongly inhibits HBV, IL-22 has only minimal antiviral activity against this virus.

To provide a more complete comparison between the cellular responses to these cytokines in hepatocytes, we performed a gene expression analysis to compare the genes induced by IFN-λ and IL-22. While a number of studies have examined genes induced by IFN-λ in hepatocyte-derived cell lines (Doyle et al., 2006; Marcello et al., 2006), gene expression data for IL-22 has been limited to keratinocytes and colonic subepithelial myofibroblasts (Andoh et al., 2005; Nograles et al., 2008; Wolk et al., 2006). We found that the profile of genes induced by IL-22 more closely resembled the profile of IL-6, and there was no overlap of the most highly induced genes between IL-22 and IFN-λ. Additionally, we found that IL-22 induced a much lower number of genes overall compared to IFN-λ. Therefore, despite their structural similarity and the fact that they share a common receptor subunit, there appears to be minimal functional similarities between these cytokines with respect to the transcriptional response in mouse hepatocytes.

Anti-HBV activity of IFN-λ2 in transgenic mice

While the antiviral activity of type III interferon is well established for many viruses in cell culture, its activity *in vivo* is less well understood. Although IFN-λ is produced in the mouse liver in response to infection, the IFN-λ receptor is expressed at only very low levels (Sommereyns et al., 2008). Furthermore, while injection of IFN-λ was shown to reduce hepatic viral titers in mice systemically infected with HSV-2 (Ank et al., 2006), it did not protect mice against infection with another hepatotropic virus, Thogotovirus (Mordstein et al., 2008). We observed only a moderate inhibition (≈50%) of HBV replication when mice were injected intravenously with murine IFN-λ2 as compared to that of mice that received murine IFN-β or IFN-γ. Based on the work of Sommereyns and colleagues, it is likely that low expression of the IFN-λ receptor in the mouse liver contributes to this finding.

The weak activity of IFN-λ2 in mice also led us to examine the stability of the human type III IFNs in serum. We found that the decreased activity of human IFN-λ2 in serum correlated with sensitivity of the cytokine to serum proteases, including thrombin. It is important to note that we were limited to testing murine IFN-λ2 in our mouse studies because mice do not encode a functional IFN-λ1 (Lasfar et al., 2006), which also makes direct comparison of the two cytokines not possible without using human cytokines in mice, which would have questionable physiological relevance. It should also be noted that although we used preparations of IFN-λ2 commercially purified from *E. coli*, human IFN-λ2 does not encode a predicted glycosylation site (Kotenko et al., 2003), so it is unlikely that glycosylation provides additional stability to the human protein. However, this may differ in mice, as secreted mIFN-λ2 and IFN-λ3 are both glycosylated (Bartlett et al., 2005; Lasfar et al., 2006).

Both IFN-α/β and IFN-γ are sensitive to degradation by proteases (Black, 2002; Cantell et al., 1992; Nelissen et al., 2003), and other cytokines such as TNF-α, IL-1 and IL-18 also require intracellular processing by proteases in order to be activated (Fantuzzi and Dinarello, 1999). Although the degradation of IFN-λ2 by proteases may be a regulatory mechanism for limiting its activity to a localized rather than system response, other regulatory mechanisms may also be important for the control of IFN-λ function *in vivo*. Interestingly, a soluble form of the IFN-λ receptor was recently described which binds to IFN-λ1 and inhibits its activity (Witte et al., 2009), further supporting the potential importance of precise regulation of this cytokine family. Additional studies would be necessary to determine the role of proteases in the systemic and/or local regulation of IFN-λ produced endogenously during natural infections in mice and humans.

Understanding the regulation of IFN-λ antiviral activity *in vivo* is of particular importance due to the potential for IFN-λ to be used therapeutically. Phase 1a and 1b clinical trials of pegylated-IFN-λ1 have demonstrated that the cytokine is well tolerated at relevant doses and capable of reducing HCV viral loads both alone and in combination with ribavirin. Regulation of IFN-λ activity by means of protease degradation and other mechanisms, such as differential receptor expression, may result in a more localized response with

fewer side effects, unlike those observed with IFN-α. Further studies are needed to fully understand the regulation and activity of IFN-λ in the antiviral response *in vivo*.

Materials and methods

Cell lines and reagents

Human hepatocellular carcinoma cells (HepG2, Huh7) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 μg penicillin/ml, 100 U streptomycin/ml, 2 mM L-glutamine, 1× MEM nonessential amino acids (Invitrogen, Carlsbad, CA), 1 mM sodium pyruvate, 10 mM HEPES buffer, and 10% heat inactivated fetal bovine serum (Invitrogen). The HepG2-derived cell line containing the HBV genome, HepG2(2.2.15), was cultured in the same manner as the HepG2 cells (Sells et al., 1987). Mouse immortalized hepatocytes (HBV-Met, TREHBV, TREHBV-V, and MMHD3) were propagated in RPMI 1640 medium containing 100 ng epidermal growth factor (BD Biosciences)/ml, 16 ng insulin-like growth factor II/ml, and 10 μg insulin (Sigma, St. Louis MO)/ml. Human IFN-λ1 and -λ2, murine IFN-λ2, IFN-γ, IL-6, and IL-22 were purchased from Peprotech (Rocky Hill, NJ). Human IFN-α(A/D) and IFN-α2a and were purchased from PBL InterferonSource (Piscataway, NJ). Mouse IFN-β was provided by Toray Industries (Chiba, Japan) and purchased from PBL. Normal pooled human serum was obtained from Innovative Research (Novi, MI), and protease inhibitors (inhibitor cocktail P-2714 and hirudin) and recombinant human thrombin were purchased from Sigma. Mouse α-thrombin was purchased from American Diagnostica (Stamford, CT).

Transgenic mice

HBV transgenic mice (strain 1.3.32) have been previously described (Guidotti et al., 1995, 1996), and were provided by Frank Chisari (The Scripps Research Institute). These animals encode a 1.3-overlength copy of the HBV genome (serotype ayw), and reproduce the virus replication cycle from gene expression through virion release. Groups of mice in all experiments were matched for age (8–12 weeks), gender, and serum HBeAg as determined by ELISA (International Immunodiagnostics, Foster City, CA) before use. All procedures were performed in accordance with the Animal Care and Use guidelines of Yale University.

HBV DNA analysis

Cells were washed in 1× PBS before the addition of 500 μl of total DNA lysis buffer (50 mM Tris–HCl pH = 8.0, 20 mM EDTA, 1% SDS) or cytoplasmic lysis buffer (150 mM NaCl, 10 mM Tris-base, pH 8.0, 0.5% Nonidet P-40). Cytoplasmic lysates were transferred to microcentrifuge tubes and nuclei were pelleted by centrifugation for 5 min at 12,000 rpm and 4 °C and supernatants were treated with 5 U of micrococcal nuclease and 1 U of DNase at 37 °C for 30 min, followed by 20 mM EDTA and 1% SDS to stop nucleases. Both total and cytoplasmic lysates were digested with 1 mg/ml proteinase K overnight at 37 °C, and DNA was extracted by phenol/chloroform extraction. Twenty micrograms of DNA were digested with EcoRV (TREHBV) or HinDIII (HBV-met and HepG2) overnight at 37 °C prior to analysis by Southern blotting with a ^{32}P-labeled HBV DNA probe. Quantifications were performed by phosphoimager analysis (Fuji Industries) using ImageGauge software.

HBV RNA analysis

Total RNA was harvested and prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA). Encapsidated RNA was extracted from cells by first washing with 1× PBS, followed by incubation in 600 μl of cytoplasmic lysis buffer on ice for 10 min. Lysates were transferred to microcentrifuge tubes and nuclei were pelleted by centrifugation for 5 min at 12,000 rpm and 4 °C. Three hundred microliters of the supernatant was used for the extraction of encapsidated RNA as previously described (Wieland et al., 2005). RNA was dissolved in 55 μl of water and 25 μl was used for Northern blotting with a ^{32}P-labeled HBV DNA probe.

Quantitative real-time PCR

Reverse transcription and quantitative real-time PCR were performed as previously described (van den Pol et al., 2007). Briefly, 1 μg of total RNA was reverse-transcribed using the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) with random hexamers. Quantitative PCR was performed in duplicate using an Applied Biosystems 7500 real-time PCR system. PCR mixtures contained 100 ng reverse-transcribed RNA, 12.5 μl SYBR green reaction mix (Applied Biosystems), and 200 nM sense and antisense primers in a total volume of 25 μl. The primer sequences used were as follows: GAPDH, 5′-AAG TAT GAC AAC AGC CTC AAG ATC-3′ (sense), 5′-CTG TGG TCA TGA GTC CTT C-3′ (antisense); MxA. 5′-ACA GGA CCA TCG GAA TCT TG-3′ (sense), 5′-CCC TTC TTC AGG TGG AAC AC-3′ (antisense); HBV, 5′-CCC GTT TGT CCT CTA ATT CC-3′ (sense), 5′-GTC CGA AGG TTT GGT ACA GC-3′ (antisense) (Yang et al., 2002). After an initial incubation at 95 °C for 5 min, PCR amplification was performed by cycling 50 times for 30 s at 95 °C followed by 1 min at 60 °C. Gene expression was quantified using the 7500 system Sequence Detection Software (Applied Biosystems) after normalization to GAPDH expression. The ΔΔCt method was used for analysis of all quantitative RT-PCRs.

STAT phosphorylation

Cells were washed once with PBS and lysed with 2× SDS sample buffer. Extracts were separated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Bio-Rad), and probed with antibodies specific for phospho-STAT-1 (Y701), STAT-1, phospho-STAT-2 (Y690) and STAT-2 (Cell Signaling). Proteins were visualized using LumiGLO chemiluminescent reagents (Cell Signaling) and a Fuji LAS-3000 cooled CCD camera.

Microarray

Immortalized murine hepatocytes (MMHD3) were differentiated for 10 days in 2% DMSO prior to treatment with 100 ng/ml of murine IFN-γ, IFN-λ2, IL-6 or IL-22 for 24 h. Total cellular RNA from two experiments was collected using the RNeasy Mini Kit (Qiagen), and RNA concentration and quality were determined by absorbance spectrophotometry and electrophoresis. Prior to the microarray analysis, control RT-PCR reactions were performed on the individual samples to confirm induced expression of GBP1, ISG15 and serum amyloid A (data not shown). cRNA was prepared according to the Illumina (Illumina, San Diego, CA) protocol at the Keck Affymetrix Resource facility at Yale University. Double stranded cDNA was synthesized from total RNA using the Illumina TotalPrep RNA Amplification Kit with an oligo (dT) T7 primer. Biotin-labeled cRNA was prepared by *in vitro* transcription from cDNA then purified with the above Amplification kit. cRNA (1.5 μg) was then hybridized to the MouseWG-6 v2 Expression BeadChip, and processing was performed following standard protocols (Illumina).

Raw microarray data was normalized using the quantile method in the beadarray package (Dunning et al., 2007). Hierarchical clustering was performed with Euclidean distance and Ward's linkage. Gene Set Enrichment Analysis (GSEA) was based on GSEA software provided by the Broad Institute using the classic enrichment statistic (Subramanian et al., 2005). Genes were ranked according to their

signal-to-noise ratio between treated and untreated cells. Perturbation and GO gene sets were obtained through the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.02.022.

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