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Transcriptomic analysis of the woodchuck model of chronic hepatitis B

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Abstract

The Eastern woodchuck (*Marmota monax*) is naturally infected with woodchuck hepatitis virus (WHV), a hepadnavirus closely related to the human hepatitis B virus (HBV). The woodchuck is used as an animal model for studying chronic hepatitis B (CHB) and HBV-associated hepatocellular carcinoma (HCC) in humans, but the lack of sequence information has hitherto precluded functional genomics analysis. To address this major limitation of the model, we report here the sequencing, assembly and annotation of the woodchuck transcriptome, together with the generation of custom woodchuck microarrays. Using this new platform, we characterized the transcriptional response to persistent WHV infection and WHV-induced HCC. This revealed that chronic WHV infection, like HBV, is associated with (i) a limited intrahepatic type I interferon response, (ii) intrahepatic induction of markers associated with T cell exhaustion, (iii) elevated levels of *suppressor of cytokine signaling 3 (SOCS3)* in the liver, and (iv) intrahepatic accumulation of neutrophils. Underscoring the translational value of the woodchuck model, this study also determined that WHV-induced HCC shares molecular characteristics with a subtype of human HCC with poor prognosis.

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Transcriptome and microarray data archiving

The complete woodchuck transcriptome data is available at the NCBI SRA under accession number SRA050380 and the microarray data has been deposited in the NCBI GEO under accession number GSE36250.

POTENTIAL CONFLICT OF INTEREST

A. Leonardo Iniguez is an employee of Roche NimbleGen, the corporation which generated (and may commercialize) the custom woodchuck microarray.

Conclusion—Our data establish the translational value of the woodchuck model and provide new insights into immune pathways which may play a role either in the persistence of HBV infection or the sequelae of CHB.

Keywords

HBV; WHV; HCC; interferon; neutrophil

Approximately 350 million individuals live with CHB, and over 500,000 people die each year due to HBV-associated liver diseases, such as cirrhosis and HCC (1). Pegylated interferon- α (IFN- α) and various nucleos(t)ides are currently licensed for the treatment of CHB, but while these therapies reduce viremia and improve long-term outcome, they rarely lead to cure (2). There is, therefore, an urgent need to develop novel antiviral therapies to achieve this goal.

Due to the difficulty in obtaining liver biopsy specimens from chronically infected patients, much of what has been learned about human HBV has been determined in animal models. Studies of acute infection in transgenic (3, 4) and hydrodynamic (5) mouse models and also in chimpanzees (6) have provided useful insights into immunological mechanisms that may contribute to the outcome of HBV infection. However, the immune correlates of cure of chronic HBV infection, and the role of host-virus interactions in persistent infection, are still ill defined. Characterization of these complex immunological phenomena has been hampered by the lack of a well characterized, immunocompetent small animal model of CHB. This therefore remains an important goal, since identification of the mechanism(s) by which HBV escapes or circumvents the host immune response will likely contribute to the identification of novel targets with potential to lead to new antiviral therapies.

The Eastern woodchuck (*Marmota monax*) is naturally infected with WHV, a hepadnavirus which is genetically closely related to human HBV, and has a disease course similar to that in HBV-infected persons (7). As such, the woodchuck has been used to study viral pathogenesis, and to evaluate antiviral and anticancer therapeutics (7). However, a notable limitation of this model is that transcriptional analysis of the woodchuck is restricted to a few hundred sequenced gene segments. To address this shortcoming, we report here the sequencing, assembly and annotation of the woodchuck transcriptome, together with the generation of woodchuck microarrays. By using this new platform, we describe the first global transcriptional analysis of persistent WHV infection and WHV-induced HCC.

EXPERIMENTAL PROCEDURES

Woodchucks and WHV infection

All experimental and surgical procedures involving woodchucks were performed under protocols approved by the Cornell University Institutional Animal Care and Use Committee. Neonatal male woodchucks were infected at 3 days of age with the same WHV7P1 inoculum containing 5×10^6 WID₅₀ of WHV strain WHV7-11. All infected woodchucks were monitored serologically through 1 year post infection and assigned to infection status groups. Uninfected animals did not have antibody to WHV core antigen (anti-WHc) and were negative for antibody to WHV surface antigen (anti-WHs) and for WHV DNA. Animals that resolved infection were all anti-WHc positive and had undetectable WHV DNA. Approximately two-thirds of the resolved animals were anti-WHs positive whereas in the remainder anti-WHs was undetectable at the time of sampling. Chronically infected animals were all anti-WHc positive and anti-WHs negative, with serum WHV DNA levels 10^{10} ge/mL (except for one woodchuck with 6.5×10^9 ge/mL), and detectable WHV surface antigen. Paired normal (non-neoplastic) and tumor (neoplastic, HCC) liver tissues were

obtained from the aforementioned chronically infected animals. Tumors were detected by repeated ultrasound examination of the liver and they were grossly identifiable at necropsy. HCC was confirmed by histological analysis of H&E stained neoplastic liver tissue sections.

RNA isolation, cDNA library construction and pyrosequencing

Total RNA was isolated from liver and peripheral blood mononuclear cells (PBMC) using the RNeasy Mini or QIAamp RNA Blood Mini kits (Qiagen Inc., Valencia, CA) according to the manufacturer's specifications, and was purified to mRNA to reduce ribosomal RNA amounts using the MRRK1010 mRNA Purification Kit (PureBiotech LLC, Middlesex, NJ). The mRNA was converted to sequencing ready cDNA following the Rapid Library Preparation Method from Roche (USM 00064 GS FLX Titanium Series, cDNA Rapid Library Preparation Method Manual, Roche Applied Sciences, Indianapolis, IN). The cDNA libraries were sequenced using the Roche 454 Genome Sequencer FLX and Titanium Chemistry following the manufacturer's recommendations (GS FLX Titanium Series Sequencing Method Manual, Roche Applied Sciences).

Microarray design and analysis

Probes were selected based on uniqueness scores and base pairs composition rules (8) and were laid out on a NimbleGen Custom Gene Expression HX3 Array. Total RNA were reverse-transcribed using SuperScript double-stranded cDNA synthesis kit (Invitrogen, Inc., Carlsbad, CA), labeled with Cy3 and then analyzed according to the manufacturer's instructions (Roche NimbleGen, Madison, WI). Quantile normalization to normalize log₂ signal intensities was obtained from scanning. Three technical replicates were performed on each cDNA for each biological sample. The expression values for each technical replicate were averaged in subsequent statistical analyses.

The gene expression data was normalized by the robust multichip average algorithm implemented in Partek Genomics Suite 6.5 (Partek, St. Louis MO), and ANOVA was used to derive lists of differentially expressed probesets. Multiple testing correction was performed using the method of Benjamini and Hochberg (9). For genes with more than one probeset, the probeset with the lowest *p*-value was selected to represent the gene. Heatmaps of the top-regulated genes or gene lists were generated from the least squares mean values per animal after normalization by the z-score for each gene's values using the R statistical program (<http://www.r-project.org>). Dendrograms were generated by unsupervised hierarchical clustering in R and principal component analysis was performed using Partek.

The enrichment of differential genes relative to the gene modules described previously (10) was calculated by a custom script within R using the humanized gene symbols for the woodchuck genes and removing any module genes that were absent from the custom woodchuck microarray. Gene Set Enrichment Analysis (GSEA) was performed as previously described (11), with ranks determined by the multiplicative product of the fold-change and $-\log(FDR)$ values for each gene. Pathway and network analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen Inc.) with on-column DNase digestion using the RNase-Free DNase Set (Qiagen Inc.). Following reverse transcription into cDNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences), samples were analyzed by real time PCR on a 7500 Real Time PCR System instrument (Applied Biosystems, Inc., Foster City, CA) using EagleTaq Master Mix with Rox (Roche Applied Sciences). Target gene expression was normalized to 18S rRNA expression. Statistical significance of difference was calculated with log-transformed data by

unpaired t-test with equal variance. The primers and probes used in this study are displayed in Supplementary Table 5.

RESULTS

Woodchuck transcriptome sequencing, assembly and annotation, and generation of custom woodchuck microarrays

To account for both tissue-specific and infection outcome-related differences in the woodchuck transcriptome (Supplementary Fig. 1), non-neoplastic liver and PBMC samples from animals chronically infected with WHV (n=3), animals that had resolved WHV infection (n=3), and uninfected control animals (n=3), were pooled by tissue type and infection status and used for pyrosequencing. The resulting 5.74 million non-rRNA sequence reads gave approximately 30x coverage of the woodchuck transcriptome (Supplementary Table 1). A combined comparative and de-novo assembly approach yielded 61,034 contiguous transcripts and singletons (Supplementary Fig. 2), and these sequences were subsequently used to design custom high-density microarrays. Microarray probesets mapped to 13,448 unique human genes (BLASTN E-value < 1e-5), which equates to approximately 65-70% coverage of the human transcriptome (Supplementary Table 2).

Chronic infection with WHV and WHV-induced HCC dramatically alter intrahepatic gene expression

The custom woodchuck microarrays were used to examine gene expression in liver, spleen and kidney tissue samples of animals chronically infected with WHV (n=13), animals that had resolved WHV infection at least 12 months prior (R; n=11; range 12-18 months), and uninfected animals (U; n=10). The liver tissue of the chronically infected animals consisted of paired neoplastic (HCC; C-H) and normal, non-neoplastic (non-HCC; C-N) samples. Histological analysis of the liver tissue from select animals demonstrated inflammatory and necrotic changes in C-N, but not in R or U, and multilobular tumors of moderate to advanced malignancy in the C-H samples (Fig. 1a).

Principal component analysis (Supplementary Fig. 3) demonstrated that persistent WHV infection and WHV-induced HCC substantially alters gene expression within the liver. In contrast, consistent with the histological analysis, uninfected animals and animals that had resolved WHV infection shared similar transcriptional signatures. Unsupervised hierarchical clustering by intrahepatic gene expression segregated all C-N from R and U (Supplementary Fig. 4) and most C-H from C-N (Supplementary Fig. 5). In contrast, there was little differential gene expression in the spleen and kidneys of these same animals, consistent with the lack of significant differential WHV RNA expression in these tissues (data not shown). Based on these initial results, we focused on detailed analysis of intrahepatic gene expression patterns.

Intrahepatic transcriptional signature of persistent WHV infection

A gene module approach based on gene co-expression patterns from multiple disease conditions (10) was used to characterize the immune cell subsets in the liver associated with persistent WHV infection. As found in the previous analyses, the modular signatures of resolved and uninfected animals were very similar (Fig. 1b, bottom left modules, R vs. U), indicating that these diverse analytical approaches provide a consistent picture of differential gene expression in woodchuck tissue. The modular signature for persistent WHV infection (C-N relative to U and R) revealed a striking increase (>25% of the transcripts in each module significantly up-regulated) in the abundance of neutrophil (Module, M2.2), plasma cell (M1.1), myeloid lineage cell (M1.5 and M2.6), B cell (M1.3), IFN response (M3.1) and cytotoxic cell (M2.1) transcripts (Fig. 1b, top left and top right modules, C-N vs. U and C-N

vs. R). Taken together with the highly significant induction of genes of the leukocyte extravasation pathway (Supplementary Figure 4), these findings indicate that there is infiltration of immune cells into the liver during persistent WHV infection. This ongoing inflammatory response to WHV has important implications for disease progression since, in addition to viral proteins such as HBx (12), chronic inflammation likely also plays a role in the development of HBV-associated HCC (13). Interestingly, these immune-related transcriptional changes were accompanied by significant down-regulation of many intrahepatic metabolic pathways, such as bile acid and steroid hormone metabolism (Supplementary Fig. 4). The reason for this relative decrease in metabolic pathways is not clear, although since hepatocyte-specific genes such as *albumin (ALB)*, *HNF-1 α (HNF1A)*, *HNF-4 α (HNF4A)* and *cytokeratin 18 (KRT18)* were not significantly modulated (absolute fold-change <1.5; data not shown), it may indicate hepatic dysfunction in persistently infected animals.

Prominent neutrophil signature in persistently infected animals

Module M2.2, which is functionally associated with neutrophils (10), was the most prominent C-N module, with 55% of the transcripts forming this module being expressed at significantly higher levels in C-N versus both R and U (Fig. 1b). Confirming the modular analysis, persistent infection was associated with a strong neutrophil transcriptional signature (Fig. 2a). Differential expression of the neutrophil granule transcripts *DEFA1*, *MMP8* and *LTF* (Fig. 2b) as well as *MPO* and *LYZ* (Supplementary Fig. 7a), was confirmed by qRT-PCR. The strong intrahepatic neutrophil signature in persistent WHV infection is noteworthy as neutrophils have been shown to play a key role in HBV immunopathology in a transgenic mouse model of acute hepatitis (14). In this mouse model, the liver damage initiated by cytotoxic T lymphocytes is amplified by non-antigen specific cells recruited into the liver via metalloproteinases (MMPs), such as MMP8 and MMP9, which are produced by neutrophils (15). *MMP8* and *MMP9* were both significantly over-expressed in C-N relative to R and U (Fig. 2a and b), as was the chemokine *CXCL9* (Fig. 3c) which, together with the neutrophil-derived MMPs, is responsible for the intrahepatic recruitment of mononuclear inflammatory cells in the aforementioned mouse model (16). Notably, liver injury in CHB patients is associated with both high serum levels of *CXCL9* (17) and accumulation of intrahepatic neutrophils (18), which suggests that comparable mechanisms play a role in the pathogenesis of WHV in woodchucks and HBV in man.

Characterization of the intrahepatic IFN signaling response in chronically infected animals

Over-representation of the IFN-inducible module M3.1 for C-N (Fig. 1b) was unexpected given that, in stark contrast to hepatitis C virus (HCV) infection (19, 20), acute HBV infection in chimpanzees (6) and man (21) is associated with a limited IFN- α/β response. However, the modular analysis did not differentiate between an IFN- α/β (type I IFNs) and IFN- γ (type II IFN) transcriptional response. The former is predominantly produced by infected cells as well as plasmacytoid dendritic cells, with the latter being primarily produced by NK cells, NKT cells and cytotoxic T lymphocytes. Examination of interferon-stimulated gene (ISG) expression revealed differential induction of type I and type II-biased ISGs in C-N. Notably, the top 10 most up-regulated intrahepatic ISGs are either induced preferentially by IFN- γ (e.g. *PLA2G2A*, *CXCL9*, *ubiquitin D (UBD)* and *GBP1*), or are induced to a similar degree by both IFN- α/β and IFN- γ (e.g. *SRGN* and *TAP1*) (Fig. 3a and b, Supplementary Table 3). In contrast, there was less up-regulation of those ISGs that are induced by type I IFN to a greater extent than type II IFN (Supplementary Table 3). An example of such is *USP18*, a negative regulator of type I IFN signaling which may play a role in modulating HBV replication (22). The strong induction of *CXCL9* relative to *USP18* was confirmed by qRT-PCR (Fig. 3c). Notably, *USP18*, but not *CXCL9*, was highly induced (up to 500-fold) in the whole blood of uninfected woodchucks after administration of

recombinant woodchuck IFN- α (Menne S and Fletcher S, unpublished data). Taken together, these data indicate that there is a strong intrahepatic type II IFN response but only a weak (or absent) type I IFN response in persistently infected animals.

Intrahepatic induction of markers associated with T cell exhaustion and inhibition of cytokine signaling in persistently infected animals

Clearance of acute HBV infection requires a vigorous, multi-specific CD8⁺ cytotoxic T cell response, whereas persistent infection is typified by limited and potentially dysfunctional (“exhausted”) T cell responses (23). There are various inhibitory receptors that can be expressed on exhausted T cells during persistent infection, including PD-1 (PDCD1) and CTLA4 (24). GSEA identified the PD-1 signaling gene set as being significantly enriched in chronically infected animals (data not shown). Consistent with this enrichment analysis, intrahepatic levels of *PD-1*, together with the ligands of this receptor, *PD-L1* (*CD274*) and *PD-L2* (*PDCD1LG2*), were significantly induced in C-N as compared to R and U (Fig. 4a). Another inhibitory T cell receptor, *CTLA4*, was also expressed at significantly higher levels in C-N relative to R and U (Fig. 4a). Importantly, these data are consistent with studies that have characterized intrahepatic T cells from CHB patients (25, 26). In addition to these negative regulatory pathways of T cell function, the intrahepatic level of *SOCS3* was also significantly elevated in C-N (Fig. 4b). *SOCS3* is a negative regulator of IL-6 (27), a cytokine which inhibits HBV replication (28), and increased intrahepatic levels of *SOCS3* protein have been observed in CHB patients (29).

Molecular characterization of WHV-induced HCC

Various genome-based classification schemes for human HCC have been developed, and they were used by us to characterize WHV-induced HCC on the molecular level. GSEA of human HCC classification signatures (Supplementary Table 4) revealed that the HCC signature in persistently infected woodchucks was positively correlated to a human HCC subclass with poor prognosis (“poor survival subclass”) and negatively correlated with a human HCC subclass with good prognosis (“good survival subclass”) (30) (Fig. 5a). The molecular features of tumor infiltrating immune cells have been shown to have prognostic value in various cancers (31, 32). Given that low level CD8⁺ T cell and NK cell infiltration is associated with poor prognosis in HCC (33), our GSEA is consistent with the marked reduction (38% of the transcripts significantly down-regulated) in the abundance of cytotoxic cell transcripts in HCC tissue (Fig. 1b, bottom right modules, C-H vs. C-N, Module M2.1). The coordinated reduction in GPCR signaling-related transcripts (Supplementary Fig. 5), likely also reflects the reduced number of immune cells in WHV-induced HCC relative to the paired non-tumor tissue (34).

WHV-induced HCC also positively correlated to the S2 subclass (Fig. 5a), a well-defined subtype of human HCC described by Hoshida and colleagues (35), which is associated with MYC activation, expression of alpha-fetoprotein (AFP) and EpCAM, and relative suppression of interferon responsive genes. Consistent with the GSEA, there was over-representation in C-H of transcriptional signatures characteristic of the MYC family (Supplementary Fig. 5 and 6). This was expected since activation of MYCN is common in WHV-induced HCC due to viral DNA integration events (36), and there was a significant increase in the abundance of *MYCN* transcripts in C-H (Fig. 5b). Further underscoring the parallels with the human S2 subclass, WHV-induced HCC was characterized by induction of both *AFP* and *EpCAM* (Fig. 5b), as well as suppression of the IFN response as evidenced by the marked under-representation of the IFN-inducible module (Fig. 1b, C-H vs. C-N Module M3.1) and the negative correlation of C-H with a human HCC subclass characterized by overexpression of select ISGs (“IFN-related subclass”) (37) (Fig. 5a). Relative suppression

of the IFN response was also consistent with over-expression of *IGF2* in C-H (38) (Supplementary Fig. 7b).

Transcriptome analysis has revealed that HBV-associated HCC is a heterogeneous disease in man, although it can commonly be classified into the “poor survival” and S2 subclasses (30, 35). Conversely, HBV-associated HCC is less frequently classified into subclasses characterized by β -catenin (CTNNB1) mutations, such as the S3 subclass (35) and the G5 subclass (39), which were negatively correlated with WHV-induced HCC (Fig. 5a). Underlining this similarity in molecular taxonomy, WHV-induced HCC was negatively correlated with the CTNNB1 subclass (Fig. 5a) which is also associated with mutations in the β -catenin gene (37). Taken together this reveals that on the molecular level WHV-induced HCC has many similarities with subclasses of human HCC, and shares molecular characteristics with HBV-associated HCC.

DISCUSSION

In this study we sequenced the woodchuck transcriptome, generated woodchuck microarrays and characterized the global transcriptional response of woodchucks to persistent WHV infection and WHV-induced HCC. Notably, the current study revealed that WHV-induced HCC shares molecular characteristics with a subclass of human HCC with poor survival (30), and with another subclass (“S2”) characterized by expression of AFP and EpCAM as well as activation of MYC (35). Although there are a wide range of animal models that have been used to study the mechanisms that drive development of HCC and to evaluate various treatment modalities (40), to our knowledge the woodchuck model is unique in that HCC develops in animals chronically infected with an hepadnavirus (7). This is an important distinction from the other models, since the woodchuck model features physiologically relevant (i.e. virus and not transgene-derived) levels of viral proteins, as well as an associated chronic inflammatory response which likely plays an important role in the development of HBV-associated HCC (13). Taken together with the molecular classification of WHV-induced HCC described in the current study, this suggests that the woodchuck model has distinct value for testing certain molecular targeted therapies for HCC. For example, consistent with activation of the IGF signaling pathway (41), *IGF2* is over-expressed and *IGFBP3* down-regulated in WHV-induced HCC (Supplementary Fig. 7b), which suggests that the woodchuck model could be used to evaluate IGF-1R blockade.

Characterization of the transcriptional response to persistent WHV infection revealed that there are important parallels between the immune response to WHV in woodchucks and HBV in man. These include the following features: (i) a limited type I interferon response (21), (ii) intrahepatic induction of *PD-1* and *CTLA4* (25, 26), (iii) over-expression of *SOCS3* in the liver (29), and (iv) intrahepatic neutrophil accumulation (18). Importantly, these data establish the translational value of the woodchuck model to derive the immunological principles of hepadnavirus persistence. While characterization of these complex phenomena will also be the focus of future studies, it is interesting to consider the therapeutic implications of the immune pathways identified in the current study with regard to the treatment of chronic HBV infection or associated liver diseases (see below).

Since WHV is sensitive to IFN- α (42), the current study suggests that the lack of a robust intrahepatic type I IFN response in chronically infected animals may contribute to viral persistence. However, since both humans (21) and chimpanzees (6) resolve HBV infection without inducing a strong type I IFN response, this pathway does not appear to be the critical determinant of progression to chronicity. In contrast to the antiviral activity of IFN- α , the current study indicates that WHV infection persists despite induction of a robust intrahepatic IFN- γ transcriptional response. IFN- γ is considered an important mediator of

non-cytolytic control of acute HBV infection in a transgenic mouse model (3, 4), and is temporally associated with resolution of acute infection in both woodchucks (43) and chimpanzees (6). Since induction of an IFN- γ response is often central to therapeutic vaccine strategies, the apparent lack of viral control by IFN- γ during persistent WHV infection may have important implications for the design and evaluation of such immunotherapeutics.

The intrahepatic expression of the inhibitory T cell receptor *PD-1* together with its ligands *PD-L1* and *PD-L2* is interesting in light of the study by Fisicaro and colleagues which suggests that blocking the PD-1 pathway is a potential therapeutic modality for the treatment of CHB (25). However, there are various inhibitory receptors that can be expressed on exhausted T cells during persistent infection (24), and the intrahepatic expression of *CTLA4*, another inhibitory T cell receptor, highlights the potential therapeutic challenge described in a recent study (26); namely functional reconstitution of HBV-specific T cell activity may require blockade of multiple, non-redundant inhibitory receptors.

The observation that the intrahepatic level of *SOCS3* is elevated in persistently infected woodchucks is noteworthy considering a recent study which suggested that targeting SOCS proteins is a promising new strategy for antiviral therapy (44). *SOCS3* is a negative regulator of cytokine signaling and is associated with evasion of the innate immune response by various viruses (45). Notably, high pre-treatment levels of *SOCS3* in the liver are a strong predictor of non-response to IFN-based therapy for patients chronically infected with hepatitis C virus (46). Intrahepatic *SOCS3* protein levels are elevated to varying degrees in patients with CHB (29), and consequently it will be interesting to determine in woodchucks whether these baseline levels negatively correlate with response to IFN treatment.

The current study also revealed that intrahepatic neutrophils are a prominent feature of chronic WHV infection. Neutrophils play a key role in immunopathogenesis of HBV in a mouse model of acute infection (14, 15), and accumulation of intrahepatic neutrophils is associated with liver injury in CHB patients (18). This indicates that comparable mechanisms may play a role in hepadnavirus pathogenesis in woodchucks and in man, and suggests that the woodchuck is a useful model to evaluate therapeutics that target neutrophil function (for example inhibitors of neutrophil elastase (47)) in the context of chronic infection.

In summary, the current study establishes the translational utility of the woodchuck model and provides new insights into various immune pathways which may play a role in HBV persistence or associated liver diseases. It is therefore anticipated that the novel platform developed in this foundational study will be used for further molecular exploration of this important animal model, and will facilitate the identification of new therapies for CHB and HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

WHV	woodchuck hepatitis virus
HBV	hepatitis B virus
CHB	chronic hepatitis B
HCC	hepatocellular carcinoma
SOCS3	suppressor of cytokine signaling 3
IFN	interferon
GSEA	gene set enrichment analysis
R	resolved animals
U	uninfected animals
C-N	chronic carrier animals, non-neoplastic
C-H	chronic carrier animals, HCC
M	Module
MMP	metalloproteinase
ISG	interferon-stimulated gene
HCV	hepatitis C virus
AFP	alpha-fetoprotein

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Figure 1. Histologic and transcriptional analyses indicate changes in the intrahepatic cellular composition in persistent WHV infection and WHV-induced HCC

(a) Representative hematoxylin and eosin (H&E) stained non-neoplastic liver tissue sections from uninfected (U), resolved (R) and chronically infected animals (C-N), together with neoplastic tissue from the same chronically infected animal (C-H). Histological analysis was performed on three animals from each group. (b) Modular analysis of comparative intrahepatic gene expression. Spot intensity (red: over-expressed; blue: under-expressed) denotes the percentage of transcripts significantly changed in each module (M) and is defined by the scale bar. The grid map at the bottom of the figure indicates the functional interpretation of each module (10), as displayed in the color-coded legend to the right. Coordinates define each module; e.g. M3.1 is row M3, column 1, which is defined as an IFN module by the legend. All genes had an absolute fold-change > 1.5 with a Benjamini-Hochberg corrected $FDR < 0.05$.

Figure 2. Persistent WHV infection is associated with a prominent intrahepatic neutrophil signature

(a) Heatmap of differentially expressed neutrophil-associated genes sorted by geometric mean fold-change of the C-N relative to U (top row represents the most highly induced gene). Columns represent samples from individual animals, and rows represent different genes. Gene names are listed on the right, with gene category indicated by the color of the adjacent box. Red and blue coloring of cells represents high and low expression levels, respectively, as indicated by the scale bars for normalized values. All genes had an absolute fold change > 1.5 with a Benjamini-Hochberg corrected $FDR < 0.05$. **(b)** qRT-PCR data expressed as fold-change relative to the mean of U. The bar height indicates the mean of each group, and the errors bars represent the standard error of the mean.

Figure 3. IFN signature in persistently infected animals is dominated by type II IFN response
(a) Expression heatmap of interferon stimulated genes (ISG), sorted by geometric mean fold-change of C-N relative to U (see also Supplementary Table 3). The top 10 most induced ISGs are listed on the right, with the fold-change in parentheses. **(b)** Customized pathway based on the Ingenuity canonical pathway for interferon signaling, including the top 10 most highly induced ISGs. Symbol shape indicates gene function, symbol color intensity indicates magnitude of differential expression, and the color of the gene name and arrow indicates ISG classification. All genes had an absolute fold change > 1.5 with a Benjamini-Hochberg corrected $FDR < 0.05$. **(c)** qRT-PCR data expressed as fold-change relative to the mean of U. The bar height indicates the mean of each group, and the errors bars represent the standard error of the mean.

Figure 4. Intrahepatic expression of markers associated with T cell exhaustion and inhibition of cytokine signaling

qRT-PCR data expressed as fold-change relative to the mean of U. The bar height indicates the mean of each group, and the errors bars represent the standard error of the mean.

Figure 5. WHV-induced HCC has close parallels with subclasses of human HCC

(a) HCC molecular subclasses enriched in C-H vs. C-N were identified by GSEA, as described in the Experimental Procedures. Heatmap columns represent samples, and rows represent mean expression values of all genes in each HCC classifier set. The title and Benjamini-Hochberg corrected FDR q -values from GSEA of each classifier set are indicated on the right. (b) qRT-PCR data expressed as fold-change relative to the mean of C-N. The bar height indicates the mean of each group, and the errors bars represent the standard error of the mean.