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Identification of an intrahepatic transcriptional signature associated with self-limiting infection in the woodchuck model of hepatitis B

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Abstract

The woodchuck model of hepatitis B virus (HBV) infection displays many characteristics of human infection and has particular value for characterizing the host immune responses during the development of chronic infection. Using the newly developed custom woodchuck microarray platform, we compared the intrahepatic transcriptional profiles of neonatal woodchucks with self-limiting woodchuck hepatitis virus (WHV) infection to those woodchucks progressing to persistent WHV infection. This revealed that WHV does not induce significant intrahepatic gene expression changes during the early-acute stage of infection (8 weeks), suggesting it is a stealth virus. At the mid-acute phase of infection (14 weeks), resolution was associated with induction of a prominent cytotoxic T cell signature. Strikingly, this was accompanied by high level expression of *PD-1* and various other inhibitory T cell receptors, which likely act to minimize liver damage by cytotoxic T cells during viral clearance. In contrast to the expression of *perforin* and other cytotoxic effector genes, the interferon- γ (IFN- γ) signaling response in the mid-acute phase was comparable to that in chronically infected adult animals. The absence of a strong IFN- α/β transcriptional response indicated that type I IFN is not a critical mediator of self-limiting infection. Nevertheless, a number of antiviral genes, including *viperin*, were differentially expressed during resolving infection, suggesting that a subset of IFN-stimulated genes (ISGs) may play a role in the control of WHV replication.

Conclusion—We identified new immune pathways associated with the clearance of hepadnavirus infection revealing novel molecular targets with potential for the therapeutic treatment of chronic hepatitis B.

Keywords

HBV; WHV; stealth virus; PD-1; viperin

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Microarray data archiving

The microarray data has been deposited in the NCBI GEO under accession number GSE37683.

Approximately 350 million individuals live with chronic hepatitis B (CHB), and over half a million people are estimated to die each year due to HBV-associated liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC) (1). Defining the immune determinants of viral clearance during acute hepatitis B (AHB) and during successful treatment of CHB will likely support the development of improved therapeutic strategies to treat HBV infection. However, since the current therapies for CHB rarely lead to cure (2), and identification of patients in the early preclinical phase of AHB is challenging, animal models have been used to characterize the immune requirements of viral control. Studies of acute HBV infection in chimpanzees have identified the central role of CD8⁺ T cells in the resolution of viral infection (3, 4), and studies in transgenic (5, 6) and hydrodynamic (7) mouse models have also identified potentially important determinants of HBV clearance. Since ethical and cost issues limit the use of chimpanzees for biomedical research and there is no small animal model of natural HBV infection, further dissection of the host-virus interactions during self-limiting infection would greatly benefit from the availability of an alternative, well characterized, immunocompetent animal model of AHB.

The Eastern woodchuck (*Marmota monax*) is naturally infected with WHV, a hepadnavirus closely related to human HBV. After experimental infection of neonatal woodchucks with WHV, 25-40% of animals resolve the infection, while the remainder become persistently infected and subsequently develop HCC (8). Therefore, the woodchuck has been used in a number of studies to characterize the immune correlates of viral control (9-12), but the lack of sequence information has previously precluded global transcriptional analysis. To address this major limitation of the model, we recently described the sequencing, assembly and annotation of the woodchuck transcriptome, together with the generation of custom woodchuck microarrays (13). Using this new platform, we initially characterized the intrahepatic transcriptional profiles of persistent WHV infection, and identified important parallels between the immune response to WHV in woodchucks and HBV in man (13). Since this first study established the translational value of the woodchuck model, we have now utilized the woodchuck microarray to characterize the immune determinants of WHV clearance during self-limiting infection.

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. The animals used in this study have been characterized previously by virologic, immunohistologic and histological markers, as well as by quantitative (q)RT-PCR analysis of select immune genes (9-11, 14). Briefly, neonatal woodchucks of both genders were infected at 3 days of age with the same WHV7P1 inoculum containing 5×10^6 WID_{50} of WHV strain WHV7-11. All infected woodchucks were monitored serologically through 1 year post infection and beyond and assigned to infection outcome groups. Uninfected animals were negative for antibody to WHV surface antigen (anti-WHs) and for WHV DNA. Animals that resolved infection were all negative for WHV surface antigen (WHsAg) and WHV DNA at 1 year post-infection, with most being anti-WHs positive. Chronically infected animals were all anti-WHs negative, with detectable serum WHV DNA and WHsAg at 1 year post-infection. For each group, the week 8 and week 14 post-infection liver samples were taken from different animals. Virologic, serologic, histologic and transcriptional characterization of the week 88 animals has been reported previously (13).

Microarray analysis

Total RNA were reverse-transcribed using SuperScript double-stranded cDNA synthesis kit (Invitrogen, Inc., Carlsbad, CA), labeled with Cy3, hybridized to the NimbleGen Woodchuck Custom Gene Expression HX3 Microarray (Roche NimbleGen, Madison, WI) and then analyzed according to the manufacturer's instructions. 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 a two-way ANOVA with disease status and time was used to derive lists of differentially expressed probesets. Multiple testing correction was performed using the method of Benjamini and Hochberg (15). For genes with more than one probeset, the probeset with the lowest *p*-value was selected to represent the gene. Following the ANOVA model, *post-hoc* contrast was used to identify genes differentially expressed between the resolving group at 14 weeks of neonatal infection relative to the chronic group at 88 weeks, while adjusting by their time-matched uninfected controls. Heatmaps of differentially expressed genes were generated by unsupervised hierarchical clustering of least square means expression values, after z-score normalization across samples. The enrichment of differential genes relative to the gene modules described previously (16) was calculated using the humanized gene symbols for the woodchuck genes and removing any module genes that were absent from the custom woodchuck microarray. These analyses were performed using R version 2.13.2 (<http://www.r-project.org>). Pathway 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 1. The primers and probes for *CTLA4*, *CXCL9*, *PD-1* (*PDCD1*), *PD-L1* (*CD274*), and *PD-L2* (*PDCD1LG2*) (13), as well as those for *PRF1*, *FasL* and *GZMB* (12), have previously been described.

RESULTS

Absence of intrahepatic transcriptional changes at week 8 post-infection suggests that WHV is a stealth virus

Custom woodchuck microarrays (13) were used to examine gene expression in the liver of woodchucks experimentally infected with WHV at 3 days of age that subsequently resolved the infection (R; n=10 at week 8 post-infection, n=7 at week 14 post-infection) or became chronically infected (C; n=5 at week 8 post-infection, n=9 at week 14 post-infection). Uninfected, age-matched animals were used as controls (U; n=5 at week 8, n=5 at week 14). Strikingly, pair-wise comparison of differentially expressed gene (DEG) counts indicated that there was no significant modulation of intrahepatic gene expression at week 8 post-infection (early-acute phase) in WHV-infected animals relative to the uninfected controls, with similar results for the resolving and chronic outcome animals (Fig. 1, week 8 R vs. U and C vs. U). Similar results were obtained by unsupervised hierarchical clustering. Using

this method, all but one of the week 8 samples from uninfected and infected animals clustered together (Fig. 2, cluster 2). This finding underscores the similarity of the transcriptional signatures of these animals in the early stage of infection (regardless of outcome) with the uninfected age-matched controls.

In contrast to the lack of significant changes in host gene expression, intrahepatic WHV RNA was readily detected in the early-acute phase of infection. In line with a previous analysis of the same animals (14), WHV RNA was significantly expressed in both prospective carriers and resolving animals at week 8 post-infection (Supplementary Table 2). The detection of viral transcripts, coupled with the expectation that the paracrine effect of cytokines produced by virus-detecting cells would amplify the transcriptional response to WHV, suggests that the lack of differential host gene expression does not reflect a lack of sensitivity to detect a response in a small percentage of infected cells. Instead, in line with a study describing the transcriptional response of adult chimpanzees to acute HBV infection (4), our data indicates that WHV is not detected by the innate immune response during the early-acute phase of neonatal infection, and therefore behaves as a stealth virus (17).

Intrahepatic transcriptional patterns at week 14 differentiate resolving animals from prospective chronic carriers

In contrast to week 8 post-infection, pair-wise comparisons at week 14 (mid-acute phase) revealed dramatic differences in the intrahepatic transcriptomic profiles of resolving animals relative to prospective carriers and uninfected controls (Fig. 1, week 14 R vs. U and R vs. C). Unsupervised hierarchical clustering segregated 5/7 resolving animals at week 14 (Fig. 2, cluster 1) from the chronic outcome and uninfected animals, while the 2 remaining resolving animals segregated with 3 animals that developed chronic infection into a separate group (Fig. 2, cluster 3). Interestingly, these 2 resolving animals displayed a significantly higher level of viremia and antigenemia as compared to the 5 resolving animals that clustered separately (Supplementary Table 3), which suggests that the transcriptional pattern of cluster 1 may be associated with viral control.

The extensive changes in the intrahepatic transcriptome in resolving animals were characterized by Ingenuity Pathway Analysis (IPA) (Fig. 2) and by a gene module approach based on gene co-expression patterns from multiple disease conditions (16) (Fig. 3). IPA revealed pronounced induction of T cell transcriptional signatures as well as a variety of other immune response pathways that indicate infiltration of immune cells into the liver during resolution of infection, which were absent in those animals that progressed to chronicity (Fig. 2). Consistent with IPA, the modular signature for resolving WHV infection (R relative to U and C) revealed a striking increase (>25% of the transcripts in each module significantly up-regulated) in the number of differentially expressed genes found in the T cell (Module, M2.8), cytotoxic cell (M2.1), plasma cell (M1.1), B cell (M1.3), myeloid lineage cell (M1.5 and M2.6), inflammation (M3.3) and IFN response (M3.1) modules (Fig. 3). These findings are consistent with results from a previous immunohistological analysis indicating higher intrahepatic counts of plasma cells, myeloid cells (monocyte/macrophages) and T cells at week 14 in the resolving compared to chronic outcome animals (9).

Transcriptional signature associated with HBV clearance in acutely infected chimpanzees is enriched in neonatal woodchucks resolving infection but to a large extent is also present in adult woodchucks with chronic infection

Wieland and colleagues previously identified a set of liver genes whose expression was associated with clearance of acute HBV infection in chimpanzees (4). The current study revealed that the majority (53/83) of these genes were also differentially expressed at week 14 during self-limiting infection in woodchucks (Supplementary Fig. 1, compare resolving

vs. uninfected week 14 “RU14” and prospective chronic vs. uninfected week 14 “CU14”). This demonstrates that resolution of acute WHV infection in neonatal woodchucks and acute HBV infection in adult chimpanzees are accompanied by a similar intrahepatic transcriptional signature. We then sought to evaluate the expression pattern of this gene signature in persistently infected adult woodchucks that had been infected as neonates (median age at gene signature analysis: 88 weeks post-infection, range: 60-112) (13), to distinguish genes uniquely induced during resolution from those induced in both resolution and chronic infection. Unexpectedly, almost all (51/53) of the genes induced during self-limiting infection were also significantly over-expressed in persistently infected adult animals (Supplementary Fig. 1, compare RU14 and chronic vs. uninfected week 88 “CU88”, green bar denotes genes significantly induced in both groups). This set of 51 genes included various IFN- γ -stimulated genes (e.g. *CXCL9*, *ubiquitin D*; *UBD*) (Supplementary Table 4). Analysis by qRT-PCR confirmed that *CXCL9* was induced to a similar magnitude in resolving neonates at week 14 and adult chronic carriers at week 88 (Fig. 5a). This suggests that the IFN- γ signaling response in the mid-acute phase of neonatal infection (10, 12) was comparable to that in adult animals with long-term established chronic infection (13).

Comparative analytical approach to identify intrahepatic genes tightly associated with WHV clearance in woodchucks

Since the previous analysis revealed that the intrahepatic transcriptional signature in neonatal resolving woodchucks shares many similarities with that of adult chronic carrier woodchucks, we reasoned that the transcriptome from chronic carriers could be used as a comparator to assist with the identification of genes and/or pathways closely associated with self-limiting infection. In order to perform this analysis, we included age-matched uninfected controls to enable a comparison of DEGs between the current study and a previous study in adult woodchucks chronically infected with WHV (13) (Supplementary Fig. 2). Notably, this approach identified genes that were selectively modulated during resolving infection in neonates (Supplementary Fig. 2, RU14 n=320) as opposed to adult chronic carriers (Supplementary Fig. 2, CU88 n=1742). In addition, in order to identify genes expressed at significantly higher levels in self-limiting infection compared to chronic carriers, a transcriptional pattern which may also be associated with viral control, a *post-hoc* analysis (see Experimental Procedures) of DEGs modulated in both groups (Supplementary Fig. 2, RU14 + CU88 n=942), was performed (data not shown). The full gene list from each group is displayed in Supplementary Table 5.

In order to validate this comparative approach, we first performed IPA and module analysis of those genes selectively enriched in adult chronic carriers (Supplementary Fig. 2, CU88). This revealed over-representation of platelet (M1.2) and neutrophil (M2.2) transcripts (Supplementary Fig. 3), which is notable since these cell types have been reported to play key roles in HBV immunopathogenesis (18, 19). In addition, an inflammation signature (M3.2) and the hepatic fibrosis pathway were also enriched in adult chronic carriers (Supplementary Fig. 3). Both of these processes are associated with active disease in CHB patients. Taken together, these data indicate that the comparative analysis of neonates and adult animals can be used to identify processes associated with a particular stage of infection and disease, and this approach was subsequently used to characterize the immune correlates of self-limiting WHV infection.

Transcriptional signature consistent with inhibition of cytotoxic T cell effector function is enriched in self-limiting infection

Modular analysis of those genes significantly induced in neonatal resolving infection (Supplementary Fig. 2, RU14) identified over-representation of cytotoxic cell (Fig. 4, M2.1) and T cell (Fig. 4, M2.8) transcripts in neonates resolving WHV infection relative to both

persistently infected neonates and established adult chronic carriers. This is consistent with the IPA (Fig. 4), as well as the strong differential expression of *perforin* (*PRFI*) (Fig. 5b) as well as *FasL* (*FASLG/CD95L*) and *granzyme B* (*GZMB*) (Supplementary Fig. 4a) during self-limiting infection. These expression data are in line with a previous analysis, which also observed that higher levels of biochemical markers of liver injury are induced in woodchucks that resolve WHV than in those animals that develop persistent infection (11). Interestingly, the expression of *GZMK*, but not other cytotoxic cell effector proteins, was significantly elevated in chronic carriers (Supplementary Fig. 4b, Week 88). This may reflect a change in the intrahepatic composition of NK and/or T cell subsets between acute and chronic infections (20).

The observation that a cytotoxic T lymphocyte (CTL) transcriptional signature was associated with self-limiting WHV infection is consistent with the central role of CD8⁺ T cells in the resolution of acute HBV infection in chimpanzees (3). In contrast, it was striking that resolution of WHV infection was accompanied by high level expression of the inhibitory T cell receptors *PD-1* (*PDCDI*) (21) and *BTNL2* (22) (Fig. 5c). The dramatic elevation of intrahepatic *PD-1* levels in animals resolving infection was paralleled by the induction of the ligands of this receptor, *PD-L1* (*CD274*) and *PD-L2* (*PDCDILG2*) (Supplementary Fig. 5a). Consistent with the induction of the *CTLA4* signaling pathway (Fig. 4), expression of the inhibitory T cell receptors *CTLA4* and *LAG3* (21) was also enhanced during self-limiting WHV infection (Supplementary Fig. 5b). High intrahepatic levels of PD-1⁺ and PD-L1⁺ cells have also been observed during acute HBV infection in man and the expression of PD-1 on HBV-specific CD8⁺ T cells may be important to limit liver damage during viral clearance (23). In line with the intrahepatic PD-1⁺ expression patterns observed in AHB and CHB in man (23), neonatal woodchucks resolving acute WHV infection had higher levels of *PD-1* and other inhibitory T cell receptors compared to chronically infected adult woodchucks, although these differences only reached statistical significance for *PD-1* and *BTNL2* (Fig. 5c, Supplementary Fig. 5b).

Enhanced expression of viperin and other IRF1-dependent genes during resolution of WHV infection

The previous analyses established that many interferon-stimulated genes (ISGs) are expressed both in chronic adult carriers as well as during self-limiting neonatal infection (Supplementary Fig. 1), suggesting they do not mediate an effective antiviral response against WHV. The aforementioned comparative analysis (Supplementary Fig. 2) revealed that a subset of ISGs were differentially expressed in self-limiting infection (Fig. 4, RU14, Module M3.1, and data not shown), and therefore may play a more direct role in viral clearance. Most notably, *viperin* (*RSAD2*), an ISG that inhibits the replication of both DNA and RNA viruses (24), was highly expressed at week 14 in resolving outcome animals (Fig. 6a). While IFN- γ can enhance *viperin* expression, it is primarily induced by IFN- α/β in the majority of cell types (24), including hepatocytes (25, 26). However, other type I IFN-response genes were not prominently induced during resolution of WHV infection (Supplementary Table 4), which suggests that, in line with resolution of acute HBV infection in humans (27) and chimpanzees (4), woodchucks control WHV infection without inducing a strong intrahepatic IFN- α/β response. Interestingly, in addition to the IRF9-dependent pathway activated by IFN- α/β , the expression of *viperin* can also be up-regulated by an IRF1-dependent pathway independent of IFN- α/β (28, 29). Consistent with the activation of this pathway, the expression of IRF1-regulated genes *GBP2* (30) and *CIITA* (31) was strongly enhanced during self-limiting infection (Supplementary Fig. 6a). *CIITA* is the master regulator of MHC class II expression (32), while *NLRC5*, which is also induced during WHV resolution (Supplementary Fig. 6b), has a similar function with regards to MHC class I transcription (33). Given their central importance to antigen presentation,

coordinated expression of these genes may play an important role in the development of an effective T cell response against WHV.

IRF1-dependent expression of *viperin* can be induced by IFN- γ (28) or by activation of peroxisome-associated MAVS adaptor protein via RIG-I-like receptors (RLR) (29). The latter pathway is noteworthy in the context of the aforementioned IFN- α/β bias of *viperin* expression in hepatocytes, but also because the expression of other peroxisomal MAVS-regulated genes (*OAS* and *IFIT* genes) (29), as well as potentiators of RLR signaling (*DDX60*, *LGP2/DHX58*) (34, 35), were strongly induced during self-limiting infection (Fig. 6b and c, Supplementary Fig. 6c). Like viperin, OASL (36), GBP2 (37), CIITA (32), DDX60 (36) and the IFIT proteins (38) have antiviral functions, and therefore may play a role in the control of WHV replication.

DISCUSSION

In this study we utilized the new woodchuck microarray platform (13) to characterize the intrahepatic transcriptional profiles of neonatal woodchucks during the early-acute (week 8) and mid-acute (week 14) phases of WHV infection in order to compare animals that subsequently resolved WHV infection to those that became persistently infected (chronic carriers). Strikingly, this revealed that, regardless of outcome, WHV infection essentially did not impact the liver transcriptome at week 8 post-infection, with fewer than 10 of the 13,448 genes analyzed being significantly modulated relative to the uninfected age-matched controls. In contrast, intrahepatic viral mRNA was detected in all early-acute phase animals infected with WHV. These data are in line with a study in adult chimpanzees acutely infected with HBV (4), and suggest that WHV behaves as a stealth virus during the early-acute phase of neonatal infection. An important caveat is that since week 8 was the earliest time point analyzed in the current study, our data cannot verify whether there is transient immune activation very early after neonatal infection, as has been reported for infection of adult animals with high doses of WHV (39) and in vitro with HBV (40).

In neonatal woodchucks that resolve WHV infection, viral load reaches a maximum at weeks 12-14 post-infection, with the peak liver injury response occurring at week 14 (11). During this mid-acute phase, the self-limiting outcome is characterized histologically by acute hepatic inflammation (9, 14) and transcriptionally by intrahepatic markers associated with immune-mediated clearance of infected hepatocytes by both cytolytic (*perforin*, *FasL*) and non-cytolytic (*IFN- γ*) mechanisms (10, 11). Using the global transcriptome approach, the current study confirmed that both cytotoxic T cell and IFN- γ transcriptional signatures are associated with the resolving outcome at week 14 post-infection. The IFN- γ transcriptional response (as measured by expression of the IFN- γ -regulated gene *CXCL9*) in this mid-acute phase was comparable to that of established adult chronic carriers. In contrast, the cytotoxic effector genes (*perforin*, *FasL* and *granzyme B*) were selectively expressed during self-limiting infection. Functional impairment (“exhaustion”) of cytotoxic T cells during chronic viral infection occurs in a hierarchical fashion, with cytolytic activity being one of the first functions compromised, and IFN- γ production being the last effector function lost (41). Taken together with the significant expression of the inhibitory T cell receptors *PD-1* and *CTLA4* (13), as well as *LAG3* and *BTLN2* in chronic carriers (Fig. 5c, Supplementary Fig. 5b), our data is consistent with the presence of dysfunctional intrahepatic T cells in persistently infected adult woodchucks.

The current study revealed that self-limiting infection was associated with a remarkable elevation (mean 75-fold) of *PD-1* at week 14, which is far greater than that observed in adult chronic carriers. Up-regulation of PD-1 on HBV-specific T cells during acute HBV infection in man has been proposed to restrain the pathogenic CD8⁺ T cell response and prevent

severe liver damage (23). The data from the current study suggest that similar mechanisms may operate to control liver immunopathogenesis during clearance of WHV from woodchucks and HBV in man. Furthermore, the induction of other T cell inhibitory receptors (*CTLA4*, *LAG3*, *BTLN2*) during self-limiting infection in woodchucks suggests that PD-1 may not have an exclusive role in tempering virus-specific T cell responses during acute hepatitis B virus infection.

While studies have indicated that there are IFN-induced cell intrinsic antiviral effectors of HBV, identification of these restriction factors has remained challenging. It is therefore noteworthy that *viperin*, an ISG with broad-spectrum antiviral activity (24), was differentially expressed (mean 24-fold) during self-limiting WHV infection. Although *viperin* is induced predominantly by IFN- α/β in hepatocytes (25, 26), our data indicated that, consistent with acute HBV infection in humans (27) and chimpanzees (4), resolution of WHV infection is not associated with a strong intrahepatic IFN- α/β response. The increased *viperin* expression was therefore not likely a result of type I IFN signaling. In contrast, this upregulation may be explained by the recent finding that *viperin* and a subset of ISGs can also be induced by an IRF1-dependent, IFN- α/β -independent pathway, either by IFN- γ (28) or by activation of peroxisome-associated MAVS adaptor protein via RIG-I-like receptors (RLR) (29). Given the previously reported IFN- α/β bias of *viperin* expression in hepatocytes, it is tempting to speculate that RLR-dependent sensing of WHV induces expression of *viperin* and other antiviral genes during self-limiting infection. Additional studies are needed to test this hypothesis. It will also be important to evaluate whether hepatitis B viruses are susceptible to *viperin* and/or the other antiviral genes that are identified here as being induced during resolving WHV infection (*OASL*, *GBP2*, *IFIT1B*, *DDX60*, *CIITA*) (32, 36-38), and also to determine whether WHV and HBV actively suppress the expression of these genes during persistent infection. In this regard, it is interesting to note that various viruses can repress IRF1-induced antiviral activities (42, 43) and also that HBV \times protein (HBx) can inhibit RLR signaling via degradation of MAVS (44).

In summary, by global transcriptional characterization of self-limiting WHV infection, this study identified new immune pathways associated with resolution and persistence of hepatitis B virus infection, and thereby provides new insights into targets with the potential for therapeutic intervention of CHB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HBV	hepatitis B virus
WHV	woodchuck hepatitis virus
IFN	interferon
ISG	interferon-stimulated gene
CHB	chronic hepatitis B
HCC	hepatocellular carcinoma
AHB	acute hepatitis B
R	resolving or resolved animals
C	(prospective) chronically infected animals
U	uninfected animals
DEG	differentially expressed gene
IPA	Ingenuity Pathway Analysis
M	Module
RLR	RIG-I-like receptor

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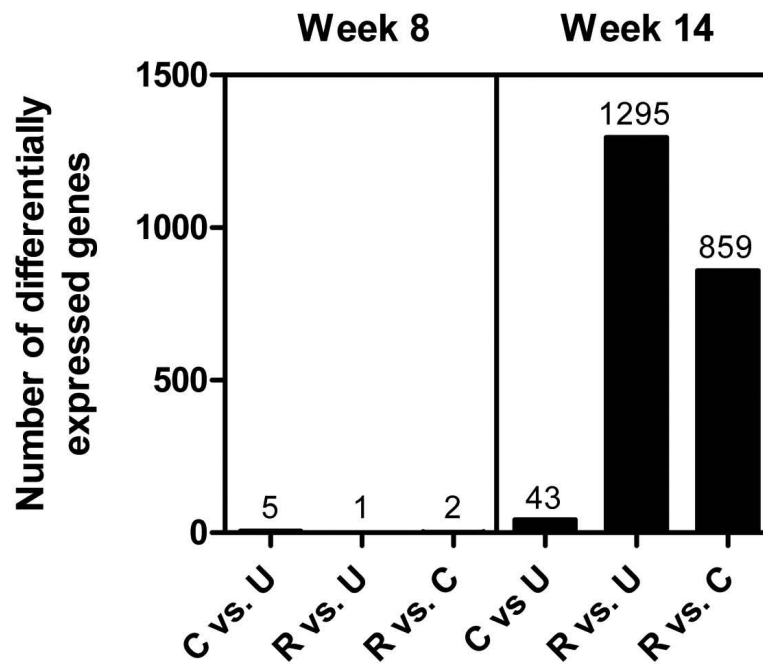


Figure 1. Pairwise comparison of gene counts by age and infection outcome

Comparison of groups resolving or progressing to chronic WHV infection relative to each other and also to the time-matched uninfected controls. The number of differentially expressed genes (DEG) is displayed above each of the pairwise comparisons. All genes had an absolute fold-change >1.5 with a Benjamini-Hochberg corrected $FDR < 0.05$. A total of 13,448 genes were analyzed.

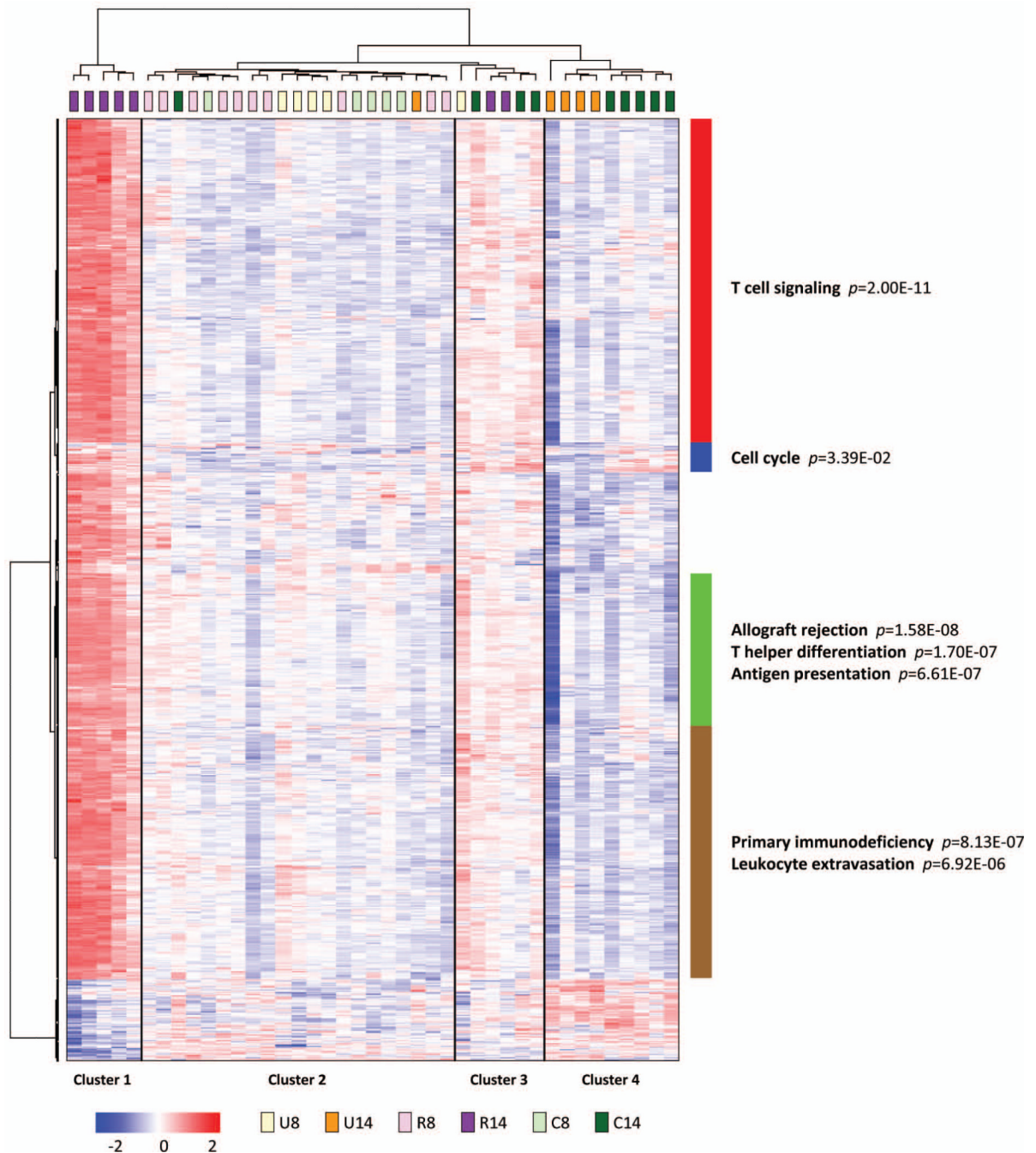


Figure 2. Resolution of WHV infection profoundly alters intrahepatic gene expression during the mid-acute phase of infection

Unsupervised hierarchical clustering of top differentially expressed intrahepatic genes of animals at 8 weeks (8) or 14 weeks (14) post-infection that went on to either resolve (R) or become chronically infected (C) with WHV, together with age-matched uninfected (U) controls. All genes had an absolute fold-change >1.5 with a Benjamini-Hochberg corrected $FDR < 0.05$. Heatmap columns represent samples from individual animals, and rows represent different genes. Red and blue coloring of cells represents high and low expression levels, respectively, as indicated by the scale bars for normalized values. Functional annotation of gene clusters was performed by Ingenuity Pathway Analysis, with the top

canonical pathways for each cluster being displayed. Pathway enrichment was calculated with the Fisher's exact test with multiple testing correction by the Benjamini and Hochberg method. Gene clusters that were not significantly enriched for a pathway were not functionally annotated.

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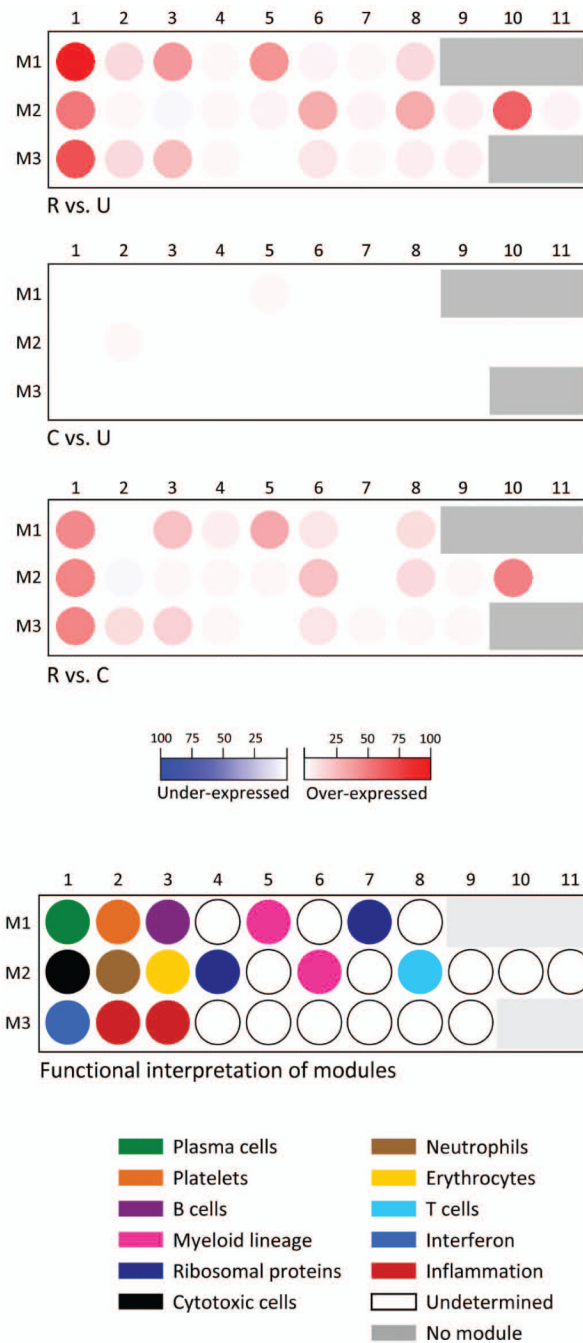


Figure 3. Transcriptional analysis indicates changes in the intrahepatic cellular composition during resolution of neonatal WHV infection

Modular analysis of intrahepatic transcriptional signatures at week 14 related to infection outcome. 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 (16), 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.

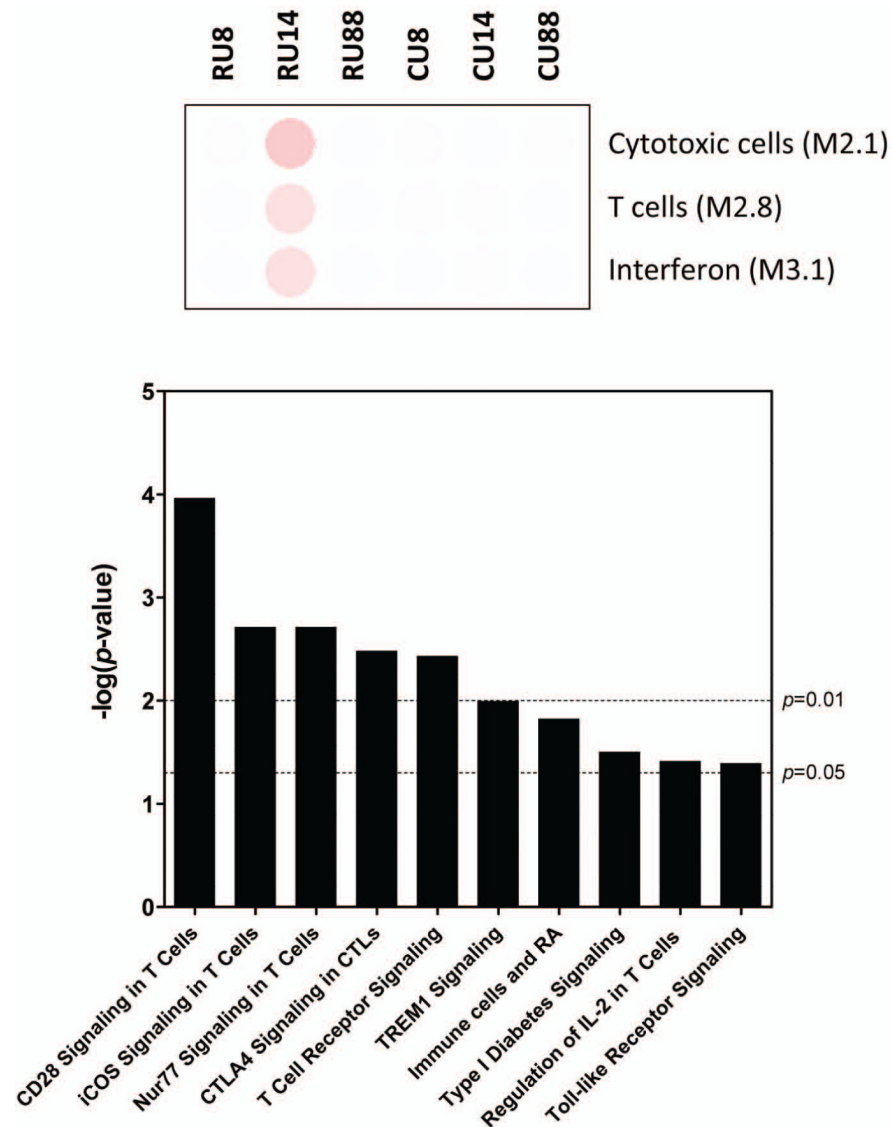


Figure 4. Characterization of intrahepatic transcriptional signature associated with resolution of WHV infection

Analysis of genes differentially induced in self-limiting infection (see Supplementary Fig. 2, “RU14”). Top panel: modular analysis of comparative intrahepatic gene expression, as described in Fig. 3. Only modules for which a significant number of genes were differentially expressed ($>10\%$ of module genes, $p < 0.05$ by Fisher's exact test) are displayed. Bottom panel: top canonical pathways identified by Ingenuity Pathway Analysis. Pathway enrichment was calculated with the Fisher's exact test with multiple testing correction by the Benjamini and Hochberg method. The $-\log(p\text{-value})$ for $p=0.05$ and $p=0.01$ significance levels are indicated.

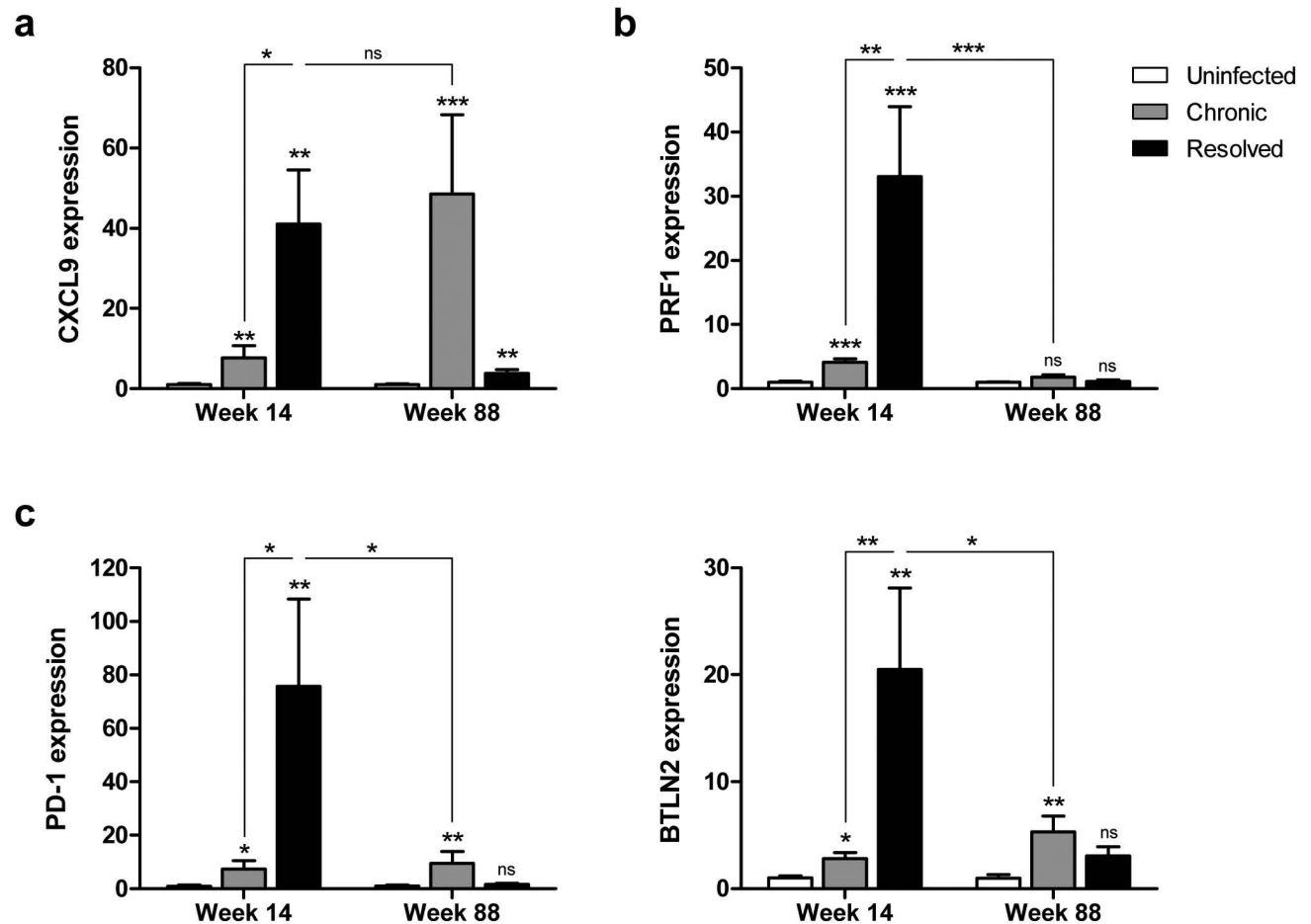


Figure 5. Intrahepatic expression of genes associated with T cell response

qRT-PCR data expressed as fold-change relative to the mean of the time-matched uninfected control. The bar height indicates the mean of each group, and the errors bars represent the standard error of the mean. The number of animals analyzed in each group was as follows: uninfected week 14; n=5, chronic week 14; n=9, resolved week 14; n=7, uninfected week 88; n=10, chronic week 88; n=11 and resolved week 88; n=11. The symbols immediately above the bars denote the level of statistical significance relative to the age-matched uninfected controls: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant ($p > 0.05$). The symbols above the horizontal lines indicate the level of statistical significance between resolving and chronic outcome animals at week 14 (left line) and between resolving outcome animals at week 14 and chronic adult carriers (right line).

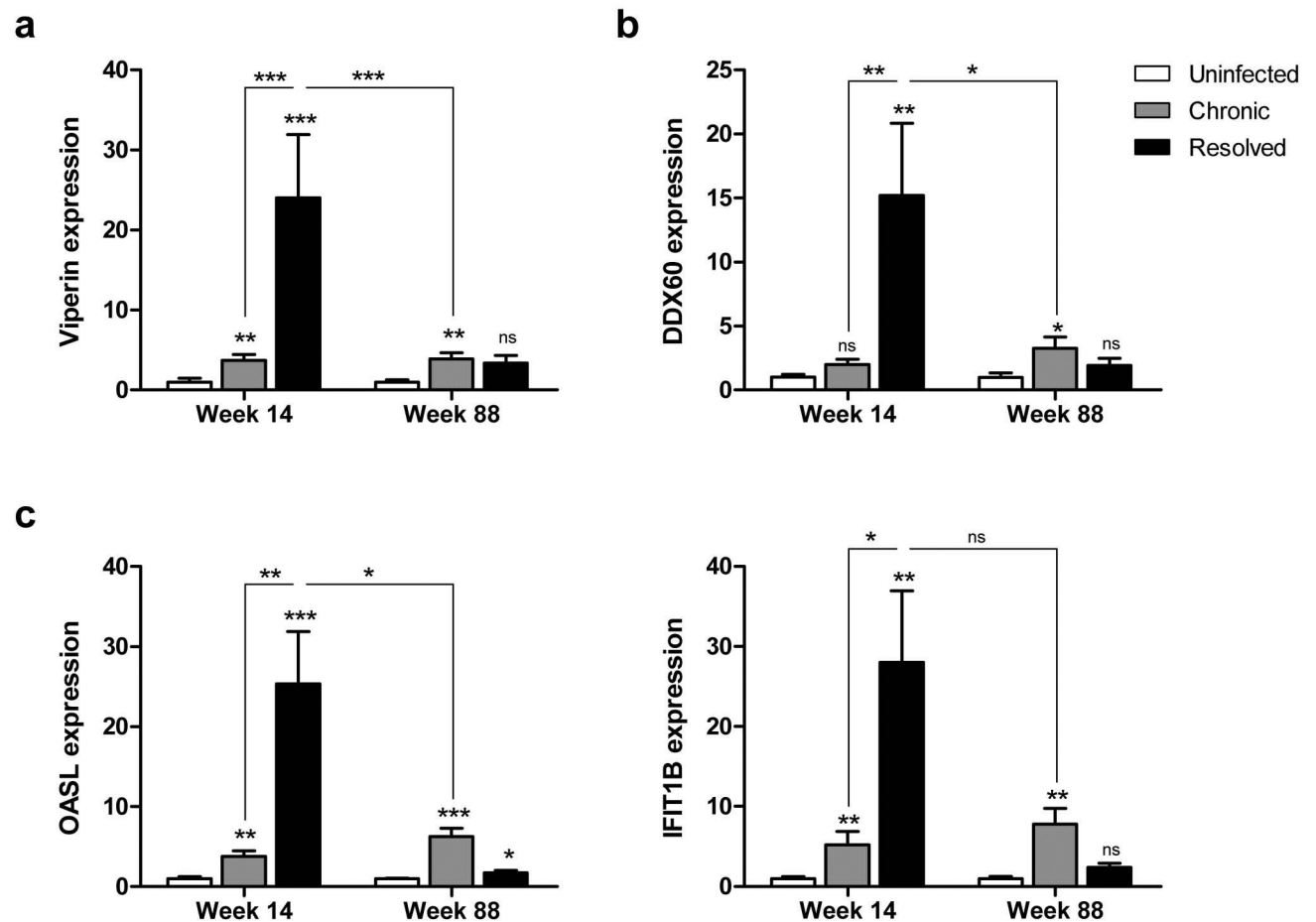


Figure 6. Intrahepatic expression of antiviral effectors associated with self-limiting WHV infection

qRT-PCR data expressed as fold-change relative to the mean of the time-matched uninfected control. The bar height indicates the mean of each group, and the errors bars represent the standard error of the mean. The number of animals analyzed in each group and the symbols denoting the levels of statistical significance are described in Fig. 5. Note that although the difference between *IFIT1B* expression levels at R14 and C88 did not quite reach statistical significance ($p=0.064$), this comparison was significant when limited to those resolving animals with low viral load, i.e. Fig. 2, cluster 1 R14 animals (see Supplementary Table 3 for details).