Retinoblastoma Protein Potentiates the Innate Immune Response in Hepatocytes: Significance for Hepatocellular Carcinoma

Jack Hutcheson,1 Ryan J. Bourgo,2,3 Uthra Balaji,1 Adam Ertel,2 Agnieszka K. Witkiewicz,1,4 and Erik S. Knudsen1,4

Cancers mediated by viral etiology must exhibit deregulated cellular proliferation and evade immune recognition. The role of the retinoblastoma tumor suppressor (RB) pathway, which is lost at relatively high frequency in hepatocellular carcinoma (HCC), has recently been expanded to include the regulation of innate immune responsiveness. In this study we investigated the coordinate impact of RB-loss on cell cycle control and immune function in the liver. We found that RB depletion in hepatoma cells resulted in a compromised immunological response to multiple stimuli and reduced the potential of these cells to recruit myeloid cells. Viral-mediated liver-specific RB deletion in vivo led to the induction of genes associated with proliferation and cell cycle entry as well as the significant attenuation of genes associated with immune function, as evidenced by decreases in cytokine and chemokine expression, leukocyte recruitment, and hepatic inflammation. To determine if these changes in gene expression were instructive in human disease, we compared our liver-specific RB-loss gene signature to existing profiles of HCC and found that this signature was associated with disease progression and confers a worse prognosis. Conclusion: Our data confirm that RB participates in the regulation of innate immunity in liver parenchymal cells both in vitro and in vivo and to our knowledge describes the first gene signature associated with HCC that includes both immunoregulatory and proliferative genes and that can also be attributed to the alteration of a single gene in vitro. (HEPATOLOGY 2014;60:1231-1240)

The tumor suppressive function of retinoblastoma protein (RB) is largely the result of transcriptional repression of cell cycle regulation genes, particularly those controlled by E2F family transcription factors,1,2 and functional inactivation of the tumor suppressor RB is a common event in nearly all human malignancy.3,4 Multiple oncogenic viruses specifically inactivate RB,5 whereas others, such as hepatitis B and C, have been speculated to inactivate RB by way of indirect mechanisms such as sequestration or nuclear export.6,7 Interestingly, expression of a large subset of immune response genes are attenuated in cancers resulting from these viruses.8

The liver is constantly exposed to pathogens and must deftly distinguish tolerable agents from pathogens. Hepatocytes are capable of binding Toll-like receptor (TLR) ligands9,10 as well as presenting exogenous antigens.11 Thus, hepatocytes play a vital role in the initiation of the immune response both directly and indirectly. Notably, cirrhosis and the transitional, early stages of hepatocellular carcinoma (HCC) are characterized by chronic inflammation,12 but as the disease progresses local immune function is suppressed.13

An increasing number of studies have demonstrated that RB is involved in immune function14-17 and expression of a large subset of immune function-related genes is down-regulated upon RB-loss in cell culture models.18 Further, RB-loss results in increased viral susceptibility, decreased TLR3 expression, reduced nuclear localization of the RELA (p65) subunit of nuclear factor kappa B (NF-κB), and diminished

Abbreviations: HCC, hepatocellular carcinoma; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RB, retinoblastoma protein; TLR, Toll-like receptor.
From the 1Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX; 2Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pa; 3Ben May Department for Cancer Research, University of Chicago, Chicago, IL; 4Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas, TX.
Received November 19, 2013; accepted May 8, 2014.
production of cytokines and chemokines including interferon-beta (IFN-β), interleukin 8, and Cxcl1.19,20 While these data suggest an important role for RB in regulating the immune response, particularly in response to viral infection, relatively few studies have investigated the impact of RB-loss on the response to specific immune stimuli and thus the exact mechanisms by which RB regulates these pathways as well as any potential relationships with the canonical role of RB in cell cycle regulation remain unclear.

Given that the regulation of innate immunity is a key role of the liver, in this report we investigate the role RB-loss plays in the hepatic immune response and examine whether this has significant relevance in the development or progression of HCC.

**Materials and Methods**

*Transfection and Stimulation of Cell Lines.* HepG2 or Huh7 cells, transfected with either shRB or shNS as previously described,21 were stimulated with lipopolysaccharide (LPS) (100 ng/mL), PMA (10 ng/mL), gadoquimod (1 μg/mL), FITC-ODN 2395 (1 μg/mL) (Invivogen, San Diego, CA), fMLP (0.2-2 μg/mL, Sigma-Aldrich, St. Louis, MO) CpG DNA, or adenovirus (1 × 10⁷ viral particles/mL) for up to 24 hours.

*Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).* For mice, primers were used at an annealing temperature of 60°C and 40 cycles. The ΔΔCT values were calculated after amplification using the Power SYBR Green reagent (Applied Biosystems) and the corresponding fold-change values were graphed. For humans, TaqMan primers for specified genes (Life Technologies) were used and multiplex qRT-PCR was performed on a BiomarkHD system (Fluidigm, San Francisco, CA). Prepared complementary DNA (cDNA) samples were preamplified for 14 cycles and resultant cDNA was then loaded on a 96 × 96 gene expression chip and run for 40 cycles.

*Flow Cytometry and In-Cell Western.* For flow cytometry, harvested cells were stained for 30 minutes with purified antibodies specific to CD16, CD74, TLR4, HLADR (Santa Cruz Biotechnology, Dallas, TX), or CD44 (BD Biosciences, San Jose, CA). Data were acquired on an LSR2 fluorescence activated cell sorter (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR). For in-cell western, cells were plated in 96-well plates and treated as indicated before fixation with methanol. Cells were probed with purified anti-CD74 antibody and donkey antigoat DyLight 800-conjugated antibody (Thermo Fisher Scientific, Rockford, IL). Relative cell numbers were quantified using Sapphire700 (Li-Cor Biosciences, Lincoln, NE) and DRAQ5 (Thermo Fisher). Staining was visualized on an Odyssey CLx (Li-Cor).

*Myeloid Recruitment and Differentiation.* Hepatoma cells were plated in 24-well plates at a concentration of 50,000 cells/well. After 24 hours, 5,000 undifferentiated, serum-starved THP-1 cells were added to the top chamber of a 0.5-micron pore transwell. Membranes were washed after 4 hours and fixed in methanol. Cells were removed from the top of the membranes by gently scraping with a phosphate-buffered saline (PBS)-wetted cotton swab and membranes were visualized following 4′,6-diamidino-2-phenylindole (DAPI) staining.

*Mice.* Mice (Rb1fl/fl) containing LoxP sites flanking exon 19 of Rb1 have been previously reported.22-24

*Ethics Statement.* All mouse care, treatment, and sacrifice were conducted using the highest standards for humane animal care in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

*Adenovirus Delivery and Liver Damage Models.* Adenoviral delivery was performed on male mice, anesthetized with isoflurane (2-chloro-2-(difluoromethoxy)2,1,1,1-trifluoro-ethane) as previously described.22 Diethylamino (DEN) and 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (3 mg/kg) were administered as previously described.23,25 Following sacrifice, livers were isolated and processed as previously described.24

*Gene Expression Array and Analysis.* Total RNA was extracted and gene expression analysis was performed as previously described24 using Affymetrix GeneChip for Mouse Genome 430 2.0 and an Affymetrix GeneChip Scanner 3000 with GeneChip Operating Software v. 3.0. Following normalization,26 gene set enrichment analysis (GSEA) was performed.27
Significant differences were determined in the TM4 MultiExperiment Viewer software package with a 1.2-fold cutoff in expression change and FDR < 25%. Ontology was done using DAVID (http://david.abcc.ncifcrf.gov/).

**Immunoblotting.** Membranes were incubated with: Santa Cruz Biotechnology: Lamin B (sc-6217), E2F1 (sc-193), p107 (sc-318), Mcm7 (sc-9966), PCNA (sc-56); Cell Signaling Technology: RB (9309).

**Immunohistochemistry and Immunofluorescence.** Five-micron sections were cut from paraffin-embedded liver specimens. Hematoxylin and eosin (H&E) staining was performed by standard methods. For immunohistochemistry, sections were heated at 55°C for 30 minutes, rehydrated, and blocked against endogenous peroxidases (Dako, Denmark) and biotin (Vector Labs, Burlingame, CA) as described by the manufacturers before overnight incubation with anti-Mac2 antibody (M3/38, Cedarlane Labs, Burlington, NC) or anti-CD74 (C-16, Santa Cruz Biotechnology). For immunofluorescence, sections were rehydrated and treated with citrate buffer before overnight incubation with anti-CD74 and visualization with Alexa fluor 546-conjugated donkey antigoat secondary antibody and DAPI counterstain (Life Technologies).

**Comparative Gene Expression Analyses.** Lesion data (GSE4108, GSE6764, GSE36376, GSE50579) were obtained from the Gene Expression Omnibus. A subset of genes herein identified as the liver specific RB-loss signature (Supporting Table 1) was used to evaluate these datasets. A median-centered gene expression profile for all genes in the signature was used to order the samples by expression and observe phenotypic trends as a function of expression gradient. Additional survival information (GSE4108) was obtained from the authors and used to compare the survival curves based on liver specific RB-loss signature expression by Kaplan-Meier analysis. Significance was determined by the log rank-\(P\) value.

**Results**

**RB Mediates Hepatocyte Immune Responsiveness.** To investigate the role of RB in innate immunity, HepG2 cells were infected with a retroviral vector containing either a short-hairpin RNA specific for RB (HepG2\textsuperscript{shRB}) or with a nonspecific sequence (HepG2\textsuperscript{shNS}).\textsuperscript{21} Whereas HepG2\textsuperscript{shNS} cells demonstrated a robust response to both LPS and adenovirus, this response was subdued in HepG2\textsuperscript{shRB} cells, as evidenced by decreased expression of cytokine and pro-inflammatory transcription factor genes (Fig. 1A). One explanation for these data would be a decreased capacity for shRB cells to detect immune stimuli. To test this, expression of multiple immunoregulatory receptors was examined by flow cytometry. Expression of all examined receptors was reduced in HepG2\textsuperscript{shRB} cells as compared to HepG2 shNS cells under basal conditions as well after stimulation with LPS (TLR4 agonist), adenovirus, or phorbol 12-myristate 13-acetate (PMA) for up to 24 hours (Fig. 1B) or after acute stimulation with gardiquimod (TLR7 agonist), CpG DNA (TLR9 agonist), or N-formyl-Met-Leu-Phe (fMLP) (Fig. 1C). Huh7 cells demonstrated a similar defect, as CD74 expression was less responsive to LPS and adenoviral stimulation in Huh7\textsuperscript{shRB} cells as compared to Huh7\textsuperscript{shNS} (Fig. 1D). Functionally, RB-deficient hepatocytes displayed less uptake of CpG DNA (Fig. 1E) and a diminished capacity for recruiting myeloid cells (Fig. 1F). Taken together, these data indicate that RB regulates the ability of hepatoma cells to respond to diverse immune stimuli at least in part by regulating their ability to detect exogenous signals and contribute to a proinflammatory milieu.

**Acute RB Deletion Deregulates Cell Cycle-Related Gene Expression In Vivo.** Adenoviral-mediated delivery of Cre-recombinase in \(RB^{fl/f}\) mice was used to determine if RB-loss led to similar immune defects in vivo. Adenoviral transduction was specific to the liver and resulted in complete recombination (Supporting Fig. 1A) and subsequent RB protein ablation as previously reported.\textsuperscript{22} RB-loss was accompanied by a rapid induction in DNA synthesis, as measured by bromodeoxyuridine (BrdU) incorporation (Supporting Fig. 1A), and increased expression of proteins corresponding with DNA replication (Supporting Fig. 1B).

**Liver-Specific RB-Loss Revealed Increased Proliferative Gene Expression and Decreased Immune Function Gene Expression.** The majority of HCC cases result from chronic viral infection and thus this model of RB-loss was selected, in part, because it accounts for viral-mediated hepatitis concomitant with the loss of RB in an approximation of what occurs during HCC progression. To focus these studies on the acute response to viral challenge and RB deletion, RNA was isolated from livers 3 days following Adeno-Cre (Ad-Cre) or Adeno-LacZ (Ad-LacZ) infection for transcriptomic analyses. Analysis by GSEA revealed up-regulation of overlapping gene ontologies related to cell cycle progression (Fig. 2A) coupled with the down-regulation of multiple ontologies related to different aspects of immune responsiveness and repair (Fig. 2B). Further examination of the resultant liver specific RB-loss gene signature (Fig. 2C,D) by gene ontology (Table 1) or Ingenuity pathway analysis
Fig. 1. RB-depleted HepG2 cells are less responsive to innate immune stimuli. (A) qRT-PCR for gene expression of cytokines, chemokines, and transcription factors associated with the response to innate stimuli. Cells were cultured in triplicate and harvested after 15 minutes to 24 hours ± LPS or adenovirus. (B) Histograms and mean fluorescence intensity (MFI) of HepG2shNS and HepG2shRB cells probed for the indicated cell surface receptor by flow cytometry. Cells were plated in triplicate and treated for 1-24 hours in the presence or absence of the indicated stimuli. Data are representative of at least three experiments. (C) MFI of CD74 expression as measured by in-cell western following 1-hour exposure to the indicated stimuli. (D) Fold-change of CD74 MFI as measured by flow cytometry in Huh7shNS and Huh7shRB cells following treatment with the indicated stimuli. Cells were plated in triplicate and treated for 1-24 hours in the presence or absence of the indicated stimuli. (E) Quantitative measurement of TLR-agonist uptake by flow cytometry. HepG2shNS, HepG2shRB, Huh7shNS, and Huh7shRB cells were cultured in triplicate with ODN 2395 for 24 hours. (F) Transwell THP-1 cell migration in response to chemotactic signals from HCC cell lines ± RB for 4 hours. Following harvest, recruited cells were stained with DAPI and counted. Data represent the mean ± SEM and were compared by Welch’s t test. *P < 0.05, **P < 0.01, ***P < 0.005.
reinforced these observations, which are also consistent with previous studies.\textsuperscript{18,30} Similar to our \textit{in vitro} data (Fig. 1B), these changes in gene expression are likely in part related to NF-κB and type-I IFN dysfunction (Supporting Fig. 3A,B).

\textbf{Liver-Specific RB-Loss Gene Signature Accurately Predicts Immune-Related Gene Deregulation In Vivo.} Next we examined gene expression patterns in mice injected with Ad-Cre or Ad-LacZ, as compared to untreated mice. Three days following administration of Ad-LacZ, expression of \textit{Ccr5}, \textit{Csf1r}, \textit{Ikbkb}, \textit{Il1b}, \textit{Il15r}, and \textit{Il2r} was increased, while Ad-Cre-treated mice displayed reduced expression of these genes (Fig. 3A). Similar results were found in the expression of \textit{Ccl2} and \textit{IgG2a} at both the mRNA and protein level (Fig. 3B). These data collectively suggest a decreased inflammatory state in Cre-positive livers as compared to Cre-negative livers, associated with the loss of RB and adenoviral-mediated hepatitis.
Histological examination of livers isolated 6 days following infection confirmed that livers from Ad-LacZ-treated mice displayed a more robust inflammatory response, measured by immune cell infiltration, as compared to livers from Ad-Cre-treated mice (Fig. 3C). This was evidenced by a significant decrease in macrophage recruitment, as determined by Mac-2 staining (Fig. 3D). Furthermore, while RB-sufficient hepatocytes, particularly those surrounding central venules, expressed high levels of CD74, this expression was largely suppressed in RB-deficient livers (Fig. 3E).

The decreased infiltration of immune cells was also present in Rb1f/f; Alb-Cre+ mice following treatment with TCPOBOP (Fig. 3F). Thus, as suggested by our in vitro data, the deregulation of hepatic immunity that results from liver-specific RB-loss extends beyond viral-mediated hepatitis in vivo.

**Murine Liver-Specific RB-Loss Gene Signature Correlates With Progression to HCC and Poor Clinical Outcome.** Following DEN injection, mice with liver-specific RB loss developed more tumors than RB-sufficient controls, as expected (data not shown); however, tumoral and peritumoral areas displayed decreased expression of CD74 (Fig. 4A). Given our previous findings and this possible association between CD74-expression and hepatocarcinogenesis, we analyzed existing human HCC gene expression datasets retrieved from the Gene Expression Omnibus31 for evidence of the liver-specific RB-loss gene signature (Supporting Table 1). This signature was able to differentiate liver tumors from adjacent nontumor tissue (Fig. 4B) and was independent of disease etiology (Fig. 4C). Subsequent GSEA revealed that while there was positive enrichment of proinflammatory genes as normal liver developed into cirrhosis, gene ontologies similar to the liver-specific RB-loss gene signature (Table 1) began to be negatively enriched at the onset of dysplasia and this trend continued as HCC progressed (Fig. 4D). These findings were further supported by examination of individual disease state gene signatures, wherein the liver-specific RB-loss gene signature was not overtly associated with cirrhosis or liver dysplasia, but demonstrated a reciprocal relationship with the progression of HCC (Fig. 4E).

These observations suggest that RB pathway disruption plays a transformative role for RB in disease progression. In sum, our findings suggest that RB-loss, at least as it relates to human disease, is likely a later stage event that promotes lesion dedifferentiation and...
aggressiveness. In accordance with this assertion, the liver-specific RB-loss signature was predictive of poor outcome in patients, with even intermediate signature expression correlating with decreased survival (Fig. 4F). Taken together, these findings indicate that RB pathway disruption, which results in both increased
Fig. 4. Liver-specific Rb-loss signature correlates with HCC progression and poor outcome in human disease. (A) Representative CD74 staining of RB-deficient (n = 5) and RB-sufficient (n = 5) livers isolated from mice 9 months after DEN treatment. (B) Clustering of patient liver samples and adjacent nontumor liver tissue (n = 433) based on liver-specific RB-loss signature expression. Gene expression boxplots represent selected RB-loss signature genes. (C) Gene expression boxplots for representative genes selected from the liver-specific RB-loss signature in normal liver tissue (n = 7) or HCC associated with alcohol abuse (n = 8), cryptogenic cause (n = 11), HBV (n = 8), or HCV (n = 9). (D) Patient liver sample GSEA grouped by disease state (n = 75). (E) Clustering of patient liver samples (n = 75) from varying disease states, based on liver-specific RB-loss signature expression. Gene expression boxplots represent selected RB-loss signature genes. (F) Kaplan-Meier plots indicating survival probability in human patients (n = 58) with High, Intermediate, or Low expression of the liver-specific RB-loss signature. *P < 0.05, **P < 0.01, ***P < 0.005.
cellular proliferation as well as decreased immune function, is a defining event for HCC progression in human disease, and could play a critical role in determining the aggressiveness and outcome of individual disease cases.

**Discussion**

Multiple reports have identified gene signatures in HCC associated with increased proliferation and decreased chromosomal stability.\(^{32}\) Meanwhile, in viral-mediated oncogenesis there is an inherent evolutionary benefit to immune evasion and a gene signature composed solely of immune-mediated genes that correlates with improved patient prognosis in early-stage HCC has recently been reported.\(^{33}\) RB-loss has been associated with the progression of HCC\(^{34}\) and in the present study we have markedly diminished RB expression both *in vitro* and *in vivo*, to examine the role of RB in hepatic immunity.

RB-loss led to decreased inflammatory responses *in vitro* (Fig. 1) and *in vivo* (Figs. 2, 3), coinciding with previous reports that have indicated a role for RB in immune signaling and pathogen clearance.\(^{14,15,19,20}\)

Gene expression analysis of RB-deficient mouse livers allowed for the identification of a liver-specific RB-loss gene signature that we have demonstrated aligns with HCC disease progression and decreased patient survival (Fig. 4). Although our signature shares some characteristics with subtype 2 HCC, originally described by Hoshida et al.,\(^ {35}\) to our knowledge this is the first report of a gene signature associated with RB-loss that aligns with cancer progression based on both increased proliferation and decreased immune function.

Interestingly, several of the receptors and chemokines we have found to be decreased upon loss of RB have previously been associated with HCC progression. Ccl5 contributes to antitumor activity and polymorphisms in either the *Ccl5* or *Ccr5* gene increases HCC susceptibility.\(^ {36,37}\) Defects in expression and allelic variations of various HLA genes, including HLA-DR, are associated with the disease.\(^ {38}\) CD74-deficient animals experience enhanced fibrosis in response to CCl\(_4\) treatment\(^ {39}\) and primary biliary cirrhosis patients display enhanced levels of circulating CD74 which neutralizes circulating macrophage migration inhibitory factor.\(^ {40}\) Additionally, the proinflammatory senescence associated secretory phenotype (SASP) promotes obesity-induced hepatic cancer.\(^ {41}\) Paradoxically, TLR4 sensing of LPS from gut microbiota is required for initiation of HCC\(^ {42}\) and increased cytokine production has been correlated with improved prognosis in early stage HCC,\(^ {33}\) while we report here that loss of RB results in decreased expression of many immune function genes (Fig. 1, Table 1). Several of these genes overlap with the previously reported immune gene signature, yet we have demonstrated that this is associated instead with progression of HCC and decreased survival. In each case, the previously reported findings were associated with early-stage events in disease development or progression, while our data suggest that the decrease in immune responsiveness associated with RB-loss becomes more prevalent as HCC progresses (Fig. 4D,E). These findings are congruent with reports suggesting that immune function is greatly diminished in advanced HCC.\(^ {13}\)

Here, we report a potential role for RB-loss as a molecular switch separating the cytokine/chemokine producing signature of early HCC from the nonresponsive immune profile of advanced HCC. This effect would occur in concert with the cell cycle effects of RB-loss and may result in a situation where cells are proliferating rapidly but can no longer be recognized by the immune system. In summary, our findings suggest that RB plays a critical role in promoting the hepatic immune response and that RB-loss may be a key checkpoint in the cancer-immunity cycle in HCC by increasing the ability of tumors to escape immune surveillance as well as by promoting cellular proliferation.

**Acknowledgment:** The authors recognize all who participated in scientific discussion and feedback related to the article, including Dr. Linda Greenbaum of Thomas Jefferson University, and especially those from the Erik Knudsen laboratory.

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Author names in bold designate shared co-first authorship.

Supporting Information

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