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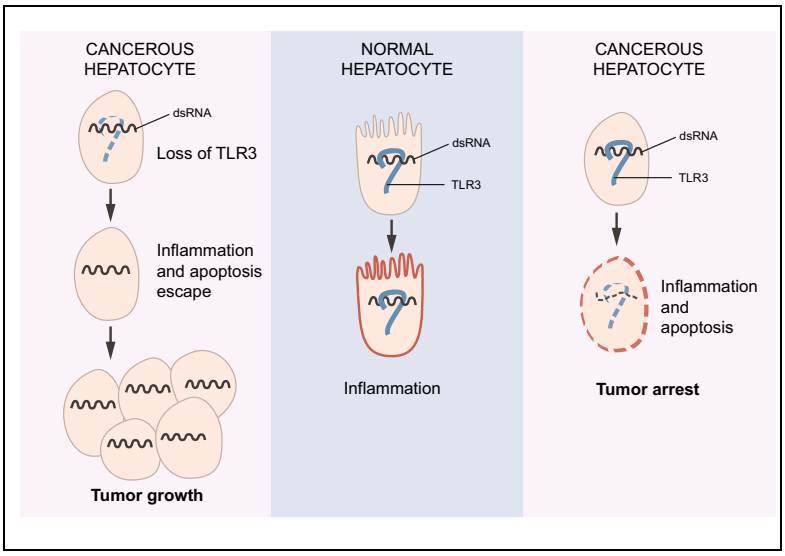
Toll-like receptor 3 downregulation is an escape mechanism from apoptosis during hepatocarcinogenesis

Graphical abstract



Highlights

- Downregulation of TLR3 in HCC is associated with poor prognosis and with resistance to TLR3-triggered apoptosis.
- Downregulation of TLR3 is an escape mechanism for HCC cells which prevents their apoptosis and enhances tumor progression.
- The effect of TLR3 on apoptosis, which limits tumor progression, is independent of the immune response.
- TLR3 ligands may represent an effective treatment option for HCC expressing TLR3.

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Lay summary

Hepatocellular carcinoma (HCC) is a heterogeneous disease associated with a poor prognosis. In patients with HCC, TLR3 downregulation is associated with reduced survival. Herein, we show that the absence of TLR3 is associated with a lower rate of apoptosis, and subsequently more rapid hepatocarcinogenesis, without any change to the immune infiltrate in the liver. Therefore, the poor prognosis associated with low TLR3 expression in HCC is likely linked to tumors ability to escape apoptosis. TLR3 may become a promising therapeutic target in TLR3-positive HCC.

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Toll-like receptor 3 downregulation is an escape mechanism from apoptosis during hepatocarcinogenesis

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Background & Aims: Low levels of toll-like receptor 3 (TLR3) in patients with hepatocellular carcinoma (HCC) are associated with poor prognosis, primarily owing to the loss of inflammatory signaling and subsequent lack of immune cell recruitment to the liver. Herein, we explore the role of TLR3-triggered apoptosis in HCC cells.

Methods: Quantitative reverse transcription PCR, western blotting, immunohistochemistry and comparative genomic hybridization were used to analyze human and mouse HCC cell lines, as well as surgically resected primary human HCCs, and to study the impact of TLR3 expression on patient outcomes. Functional analyses were performed in HCC cells, following the restoration of TLR3 by lentiviral transduction. The role of TLR3-triggered apoptosis in HCC was analyzed *in vivo* in a transgenic mouse model of HCC.

Results: Lower expression of TLR3 in tumor compared to non-tumor matched tissue was observed at both mRNA and protein levels in primary HCC, and was predictive of shorter recurrence-free survival after surgical resection in both univariate (hazard ratio [HR] 1.79; 95% CI 1.04–3.06; $p = 0.03$) and multivariate analyses (HR 1.73; CI 1.01–2.97; $p = 0.04$). Immunohistochemistry confirmed frequent downregulation of TLR3 in human and mouse primary HCC cells. None of the 6 human HCC cell lines analyzed expressed TLR3, and ectopic expression of TLR3 following lentiviral transduction not only restored the inflammatory response but also sensitized cells to TLR3-triggered apoptosis. Lastly, in the transgenic mouse model of HCC, absence of TLR3 expression was accompanied by a lower rate of preneoplastic hepatocyte apoptosis and accelerated hepatocarcinogenesis without altering the tumor immune infiltrate.

Conclusion: Downregulation of TLR3 protects transforming hepatocytes from direct TLR3-triggered apoptosis, thereby contributing to hepatocarcinogenesis and poor patient outcome.

Lay summary: Hepatocellular carcinoma (HCC) is a heterogeneous disease associated with a poor prognosis. In patients with HCC, TLR3 downregulation is associated with reduced survival. Herein, we show that the absence of TLR3 is associated with a lower rate of apoptosis, and subsequently more rapid hepatocarcinogenesis, without any change to the immune infiltrate in the liver. Therefore, the poor prognosis associated with low TLR3 expression in HCC is likely linked to tumors ability to escape apoptosis. TLR3 may become a promising therapeutic target in TLR3-positive HCC.

Introduction

Hepatocytes are constantly exposed to chemicals, activated by bacterial products, and frequently infected by viruses. These assaults can lead to chronic hepatitis or cirrhosis, conditions associated with hepatocarcinogenesis. Similarly to other cells, hepatocytes defend themselves against viruses by initiating an innate immune response resulting in the production of type I interferons endowed with anti-viral activities.[1] Activation of the innate immune system depends on the detection by Pattern Recognition Receptors (PRRs) of molecular cues signaling the presence of microbes.[2] Among Toll-Like Receptors (TLRs), which represent the largest family of human PRRs, TLR3 is expressed in the lysosomes of various types of cells, including immune cells, epithelial cells, and hepatocytes. TLR3 recognizes double-strand RNA (dsRNA)[3] of viral[4] origin but also self dsRNA,[5] and activates NF-kB-, JNK-, AP-1-, and IRF3-dependent signaling pathways in normal cells. In the liver, recognition of HCV dsRNA replicative intermediates by TLR3 protects human hepatocytes *in vitro*,[6,7] but there is no direct evidence of its involvement in viral hepatitis. Aside from the inflammatory response, TLR3 can also trigger apoptosis in cancer cells.[8–12] We previously described the signaling complex that physically recruits caspase 8 to TLR3 and

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drives cancer cell death while sparing normal epithelial cells,[13] and a former publication suggests that TLR3 signaling could be "skewed to apoptosis" in hepatocellular carcinoma (HCC).[14] In this context, poor prognosis associated with low *TLR3* mRNA in HCC and decreased expression of the protein by human transformed hepatocytes have previously been reported. Indeed, Chew *et al.* proposed a model wherein TLR3 expressed by parenchymal and non-parenchymal liver cells, including natural killer (NK) cells, drives the intratumoral recruitment of T cells that consequently kill cancer cells.[15] In the present study, we investigate the expression of TLR3 in cancer hepatocytes and its involvement in apoptotic signaling, showing that TLR3 suppression acts as an escape mechanism from apoptosis, thus leading to enhanced hepatocarcinogenesis.

Materials and methods

Origin and analysis of cell lines and patient samples

Human cell lines were purchased from the ATCC except HepaRG,[16] FOCUS and HuH-7 that were maintained in our laboratory. We also purchased the mouse Hepa1-6 (ATCC® CRL-1830™, C57L) and MH-22A (ECACC 96121721, C3HA) cells. We had previously generated the LL-11 HCC cell line (C57Bl/6 X DBA/2).[17] Primary human hepatocytes (PHHs) and normal liver tissues (n = 11) were isolated from surgical resections of liver metastases developing from primary colorectal adenocarcinoma at the Centre Léon Bérard (France). Tumor samples (n = 126) for *TLR3* mRNA and clinical data analyses were collected and performed at the Centre Français de Ressources Biologiques (ministerial agreements #AC-2013-1871 and DC-2013-1870). All of the patients had given their written informed consent in compliance with requirements of the local ethical committee of each institution.

HCC mouse models

All animal experiments, within this study, were performed according to animal protocol (A 69383 1201) which was approved by the Lyon SFR animal ethic committee (C2EA-15: CECCAPP). Mice (5 in 1 cage) were provided free access to food and water and kept in a colony room on a 12:12 h light and dark cycle. The SV40 mouse model of HCC (AsVB, B6D2F1 hybrids) has been described previously.[18] SV40 (male) and *TLR3* wild-type (WT) or *TLR3*$^{-/-}$ (female) mice (both C57BL/6) were crossed. F1 male mice were systematically sacrificed at 8 (4 *TLR3* WT and 5 *TLR3*$^{-/-}$), 10 (8 *TLR3* WT and 7 *TLR3*$^{-/-}$) and 12 weeks of age (7 *TLR3* WT and 6 *TLR3*$^{-/-}$). Liver were examined macroscopically under 2× magnifying glasses. The right lobe of the liver was fixed in 10% buffered formalin and included in paraffin for histological analysis. For the diethylnitrosamine (DEN) HCC mouse model, a single dose of 25 mg/kg of DEN was injected intra-peritoneally at day 15 in C57BL/6 *TLR3* WT or *TLR3*$^{-/-}$ male mice (n = 10). Mice were sacrificed at 9 months. Livers were harvested and fixed in 10% buffered formalin and included in paraffin.

Immunohistochemistry and quantitative analysis of tissue sections

Formaldehyde-fixed paraffin-embedded (FFPE) tissue sections from human HCC (obtained from the Pathology Department of the Groupement Hospitalier Lyon Nord, Hospices Civils de Lyon) and from livers of 4 month-old AsVB transgenic mice[18] expressing the SV40-TAg under the anti-thrombin III promoter were stained with antibodies specific for TLR3, cleaved caspase 3, Ki67, mCD3 and mCD8 as detailed in the supplementary materials and methods. Expression of TLR3 in tumor compared to non-tumor tissues was scored semi-quantitatively as follows: the percentage of hepatoma cells expressing no, less, equal or more TLR3 than peritumoral hepatocytes was counted in 10 random fields at 100x magnification and multiplied by a factor of 0, 1, 2 or 3, respectively. A sample with the same TLR3 expression in tumoral and peritumoral tissue was scored 200. Values ranging <160 and >240 were considered to be "low" and "high" values, respectively. For mouse HCC, 4 μm thick FFPE tissue sections were obtained from 3 random cutting planes from the right liver lobe. All staining was performed with the automated immunostainer (Ventana Discovery XT, Roche, Meylan, France) using the DABmap Kit according to the manufacturer's instructions. All quantifications were performed blinded to the status of TLR3. Image analyses were performed under light microscope (Eclipse E400, Nikon France, Champigny, France) equipped with a tri-CDD video camera (Sony, Japan). The total surface of the liver section was determined by morphometric analysis (Histolab, Microvision Instruments, Evry, France). The median number of nodules was determined on H&E stained sections and reported to the total surface analyzed. Median size of nodules was automatically quantified by morphometric analysis and expressed in mm^2. Cleaved caspase 3 quantification was performed on at least 50 fields (100× magnification) randomly chosen on non-tumoral liver sections and reported to the total surface analyzed. Percentage of Ki67$^+$ cells was determined automatically by morphometric analysis on 50 randomly chosen non-tumoral fields (median of 40,000 nuclei analyzed). CD3$^+$ and CD8$^+$ lymphocyte quantification was measured on 50 fields randomly chosen on liver sections and reported to the total surface of the fields determined by morphometric analysis and presented as CD3 and CD8 density.

TLR3 mRNA quantification

TLR3 mRNA purified from tissue biopsies was quantified by quantitative reverse transcription PCR (RT-qPCR) with the Lightcycler 480 II apparatus (Roche Applied Science, Penzberg, GmbH) as detailed in the Supplemental Methods, and compared to the 50S ribosomal protein L15 (RPLO) for the study of the cell lines and to the 18S ribosomal RNA (18S) for the clinical samples, using the ΔΔCt method as described by the manufacturer. The *TLR3*/18S value for normal livers (n = 8) was 4,093 ± 863 (mean ± SEM). *TLR3* mRNA expression < mean level in normal liver − 2 SEM (TLR3/18S <2,366) was recorded as low.

Statistics

Kaplan-Meier univariate survival analysis was performed and hazard ratios (HR), 95% CIs, and *p* values were calculated using the log-rank (Mantel–Cox) test. Paired *t* tests, Mann-Whitney *U* test, Fisher's exact, Wilcoxon matched-pairs signed rank test and two-ways analysis of variance tests were also used in experiments as indicated. All statistical tests were two-tailed.

Other methods

Additional information regarding the cell culture conditions, online data from normal and HCC livers, reagents, inducible lentiviral vectors, *TLR3* mRNA quantification, Western blots, flow cytometry analysis, the cytokines/chemokines dosage,

and immunohistochemistry are described in the supplementary CTAT table and supplementary materials and methods (available online).

Results

TLR3 is frequently downregulated in human primary HCC

Quantitative comparison of TLR3 protein expression by western blot in non-tumoral versus corresponding tumoral liver samples showed highly significant suppression of TLR3 in 30 tumors (Wilcoxon matched-pairs signed rank test, $p < 0.0001$) (Fig. 1A and Fig. S1A). We then used a reliable protocol[19] to determine which cells had lost their TLR3 expression in HCC. In normal liver, all hepatocytes showed a homogeneous and punctuated pattern of cytoplasmic staining compatible with TLR3 localization in endolysosomes (Fig. S1B, upper pictures). A strong TLR3 signal was also observed in biliary epithelial cells (Fig. S1B, lower left picture). However, no other cell type expressed TLR3. In HCC (Fig. 1B), staining of transformed hepatocytes was heterogeneous, displaying the punctuated pattern seen in normal liver or an enhanced signal close to the nucleus. Like in normal liver, biliary epithelial cells were strongly TLR3$^+$ in all tumors, while inflammatory cells were negative (Fig. S1B, lower right picture). In agreement with our western blot data, with Human Protein Atlas data (see supplementary materials and methods) and with previous reports,[20–22] semi-quantitative assessment by immunohistochemistry (IHC) in tumor versus matched non-tumor tissues also revealed that TLR3 expression decreased in transformed compared to non-transformed peritumoral hepatocytes in 11/20 HCC samples (Table S1). Online data from microarray (EBI,[23] n = 75) and RNAseq (TCGA, n = 50) analyses demonstrated that *TLR3* mRNA expression is also significantly lower ($p < 0.0001$) in human HCC compared to normal liver (Fig. S1C and D, respectively). Interestingly, such a downregulation is observed neither for TRIF (the adapter shared by TLR3 and TLR4), nor for the 2 cytoplasmic dsRNA receptors RIG-I and MDA-5. These data prompted us to perform RT-qPCR to analyze the expression of *TLR3* mRNA in 126 primary HCC from patients who had undergone tumor resection (French Cohort features are shown in Table S2). Compared to normal liver, *TLR3* mRNA was expressed at similar or higher level in 80% of the samples (hereafter referred to as the "TLR3 non-low" group), but significantly downregulated (cut-off value < [mean value − 2SEM] of 8 normal livers) in 26/126 tumors (20%). The downregulation of *TLR3* mRNA was not associated with etiology (χ^2 test, $p = 0.70$, data not shown). Patients with a low *TLR3* mRNA expression had a significantly shorter recurrence-free survival (RFS) (Fig. 1C) with a median RFS for TLR3-low cases of 16 months versus 33 months in the TLR3 non-low group (Log-rank test, $p = 0.01$). Moreover, low TLR3 expression was a prognostic factor for early tumor recurrence in univariate analysis (HR 1.79; CI 1.04–3.06; $p = 0.03$) and remained independent in multivariate analysis (HR 1.73; CI 1.01–2.97; $p = 0.04$). (Table 1). Next, we analyzed the correlation between the *TLR3* mRNA level and RFS for 306 patients with HCC from the TCGA series (see supplementary materials and methods) who had undergone tumor resection and for which follow-up data was available. By

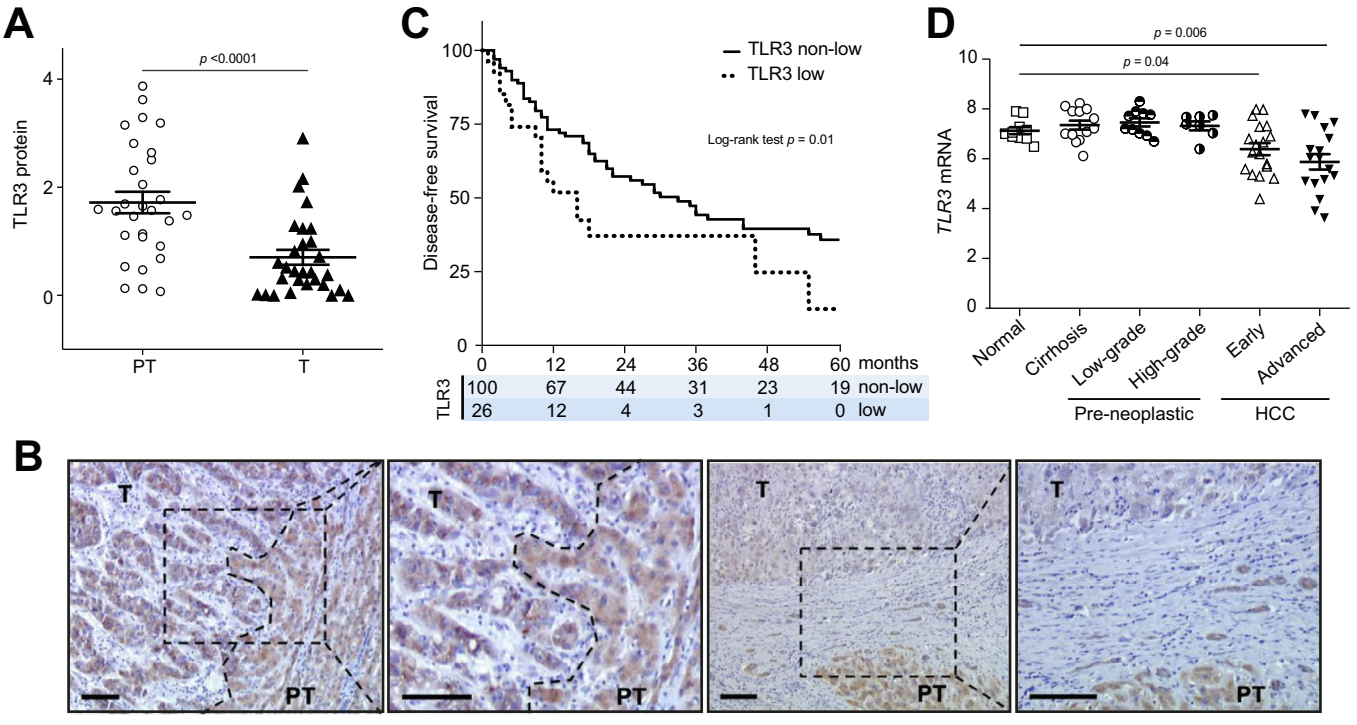

Fig. 1. Expression and prognostic value of TLR3 in human HCC. (A) Relative expression of TLR3 *vs.* the actin protein determined by western blot in 30 pairs of peritumoral (PT) *vs.* corresponding tumoral tissues (T) from the French Cohort of HCC. (B) Representative images of TLR3 expression detected by IHC in human HCC: two left pictures show the interface between peritumoral (PT) and tumoral (T) tissues in a TLR3$^+$ HCC. Two right pictures show TLR3^{low} HCC. Bars correspond to 100 μm. Original magnification: 100× (left) and 200× (right) for each HCC. (C) DFS of 126 patients with HCC of the French cohort according to the level of *TLR3* mRNA in the tumor relative to normal liver. (D) Comparison of mRNA expression of *TLR3* in normal, cirrhotic, preneoplastic and cancerous livers according to the EBI HCC cohort data available online (see results; n = 75). Statistics: A = Wilcoxon matched-pairs signed rank test; C = Log-rank test; D = two-tailed unpaired *t* test. DFS, disease-free survival; HCC, hepatocellular carcinoma; IHC, immunohistochemistry. (This figure appears in colour on the web.)

Table 1. Univariate and multivariate analysis of clinical, pathological, and molecular variables for disease-free survival for patients treated by surgical resection as first line therapy (n = 126).

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	Wald test *p* value	HR (95% CI)	Wald test *p* value
Sex (male)	1.08 (0.62–1.88)	0.77		
Age (>60 years)	0.99 (0.97–1.01)	0.23		
Etiology (HBV)	1.31 (0.77–2.24)	0.30		
Etiology (HCV)	0.64 (0.37–1.10)	0.10		
Etiology (alcohol)	1.66 (0.66–1.92)	0.54		
Etiology (NASH)	1.31 (0.77–2.22)	0.32		
Cirrhosis	1.09 (0.69–1.71)	0.72		
Tumor size >50 mm	**1.84 (1.15–2.95)**	**0.01**	**1.80 (1.12–2.90)**	**0.02**
Microvascular invasion	1.12 (0.69–1.79)	0.64		
AFP >400 ng/ml	1.12 (0.63–2.02)	0.69		
Presence of satellite nodules	1.45 (0.88–2.39)	0.10		
Poor differentiation (OMS classification)	1.24 (0.91–1.68)	0.16		
Low *TLR3* mRNA	**1.79 (1.04–3.06)**	**0.03**	**1.73 (1.01–2.97)**	**0.04**

AFP, alpha-fetoprotein; HR, hazard ratio; NASH, non-alcoholic steatohepatitis.
Patients with early perioperative mortality after resection (<2 month), as well as patients treated by liver transplantation were removed from the initial cohort. Cox proportional hazards regression test was used for univariate and multivariate analysis. Parameters with *p* value ≤0.1 in univariate analysis were included in the multivariate model.

analogy with results described above, we confirmed that the 20% patients with HCC (n = 60) and the lowest *TLR3* mRNA level had a significantly shorter RFS (Log-rank test, $p = 0.006$) (Fig. S1D). A similar result was observed using a cut-off of 50% to separate TLR3^{high} from TLR3^{low} HCC (Log-rank test, $p = 0.002$, data not shown). The transcription factor, p53, known to enhance *TLR3* transcription,[24] is the second most frequently mutated gene in HCC, occurring in about 30% of cases,[25] and *TP53* mutations are associated with poor prognosis in HCC.[26] Although the *TP53* mutation and low level of *TLR3* mRNA statuses were correlated (Fischer's exact test, p = 0.027), these 2 parameters remained independent prognostic factors of RFS in multivariate analysis once tumor size had been omitted, owing to its prevailing influence (*TP53*: HR 1.55; 95% CI 1.12–2.15; $p = 0.009$; *TLR3* mRNA: HR 1.55; 95% CI 1.13–2.13; $p = 0.007$). Lastly, closer analysis of the EBI data[23] indicated that the loss of *TLR3* mRNA expression occurred only after transformation and, as previously reported,[15] increased during disease progression (Fig. 1D). Altogether, our results confirm that TLR3 down-regulation occurs frequently in HCC cells and show that low *TLR3* mRNA expression in HCC is an independent prognostic factor for shorter RFS after surgery. The progressive loss of *TLR3*

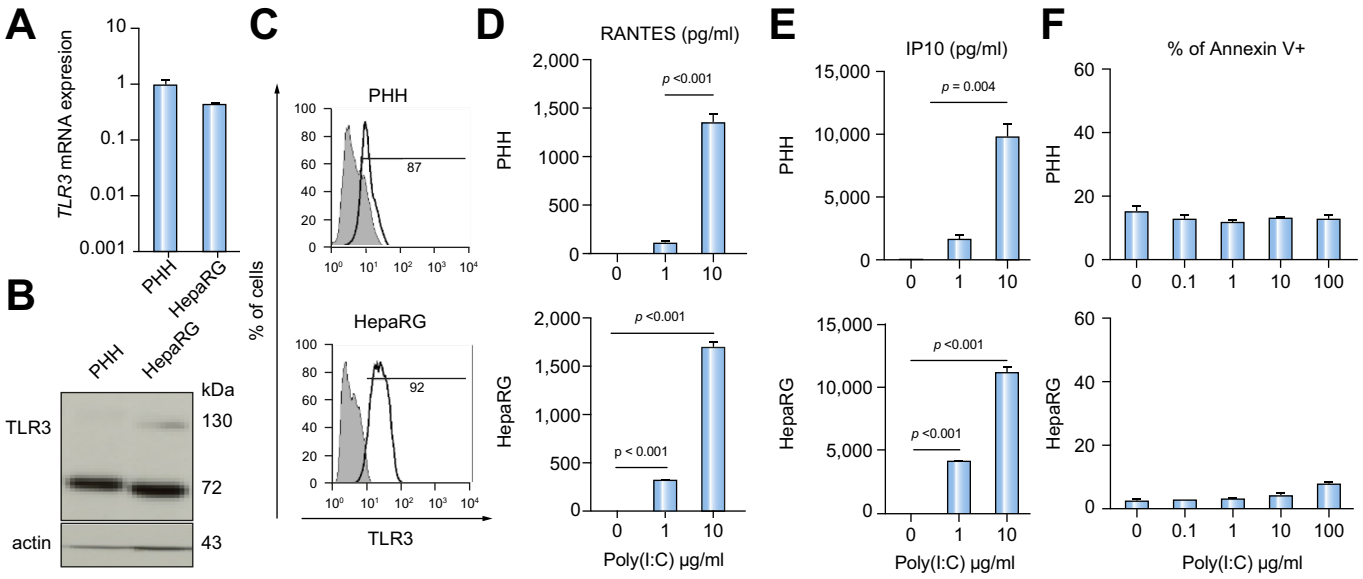

Fig. 2. Expression of TLR3 and response to Poly(I:C) in normal human hepatocytes. (A) *TLR3* mRNA expression in unstimulated PHH and HepaRG cells. (B) Western blot analysis of TLR3 expression by unstimulated PHH and HepaRG cells. (C) FACS analysis of intracellular TLR3 expression by unstimulated PHH and HepaRG cells. Grey = control isotype Ab. Numbers indicate the percentage of TLR3^{+} cells. (D, E) PHH and HepaRG cells were treated for 24 h with the indicated concentrations of Poly(I:C) and the concentration of RANTES and IP-10 in the supernatant was measured by ELISA. (F) At the end of the experimental culture periods described above, the percentage of Annexin V^{+} apoptotic cells was measured by flow cytometry. (A-C) Data are representative of 3 independent experiments. Means ± SEM of 3 independent experiments are shown (D-F). Statistics = two-tailed unpaired *t* test (D, E). Ab, antibody; PHH, primary human hepatocytes.

mRNA during hepatocarcinogenesis led us to investigate the functions of the receptor in normal and transformed hepatocytes.

TLR3 triggers an inflammatory signal in normal human hepatocytes

We used PHHs and the non-transformed hepatocyte progenitor cell line HepaRG,[16] which had been shown to respond to Poly(I:C),[27] to study the expression and function of TLR3 in normal hepatocytes. In agreement with Luangsay *et al.*,[27] PHH and HepaRG expressed similar levels of *TLR3* mRNA (Fig. 2A) and protein by western blot (Fig. 2B) and flow cytometry (Fig. 2C). Western blot analysis revealed that while they both expressed the 72 kDa C-terminal fragment of the cleaved-functional receptor,[28] PHH alone expressed the 130 kDa full length TLR3 (Fig. 2B). In response to the synthetic TLR3 ligand Poly(I:C), PHH and HepaRG both secreted comparable amounts of RANTES and IP10, 2 chemokines mostly dependent on the transcription factors NF-kB and IRF, respectively (Fig. 2D, E), while they were completely resistant to TLR3-triggered apoptosis (Fig. 2F).

TLR3 is absent from human HCC cell lines

In contrast, *TLR3* mRNA expression was very low compared to normal hepatocytes in 6/6 HCC cell lines analyzed (Fig. 3A), and the protein could not be detected either by flow cytometry (Fig. 3B) or by western blot in all HCC cell lines (Fig. 3C). Of note, the absence of TLR3 was not correlated with the p53 status of the cell lines, as SK-Hep-1 and HepG2 cells express WT p53 while the HuH7 and PLC/PRF/5 cells harbor mutated p53 and Hep3B and FOCUS cells have both alleles deleted.[29,30] In

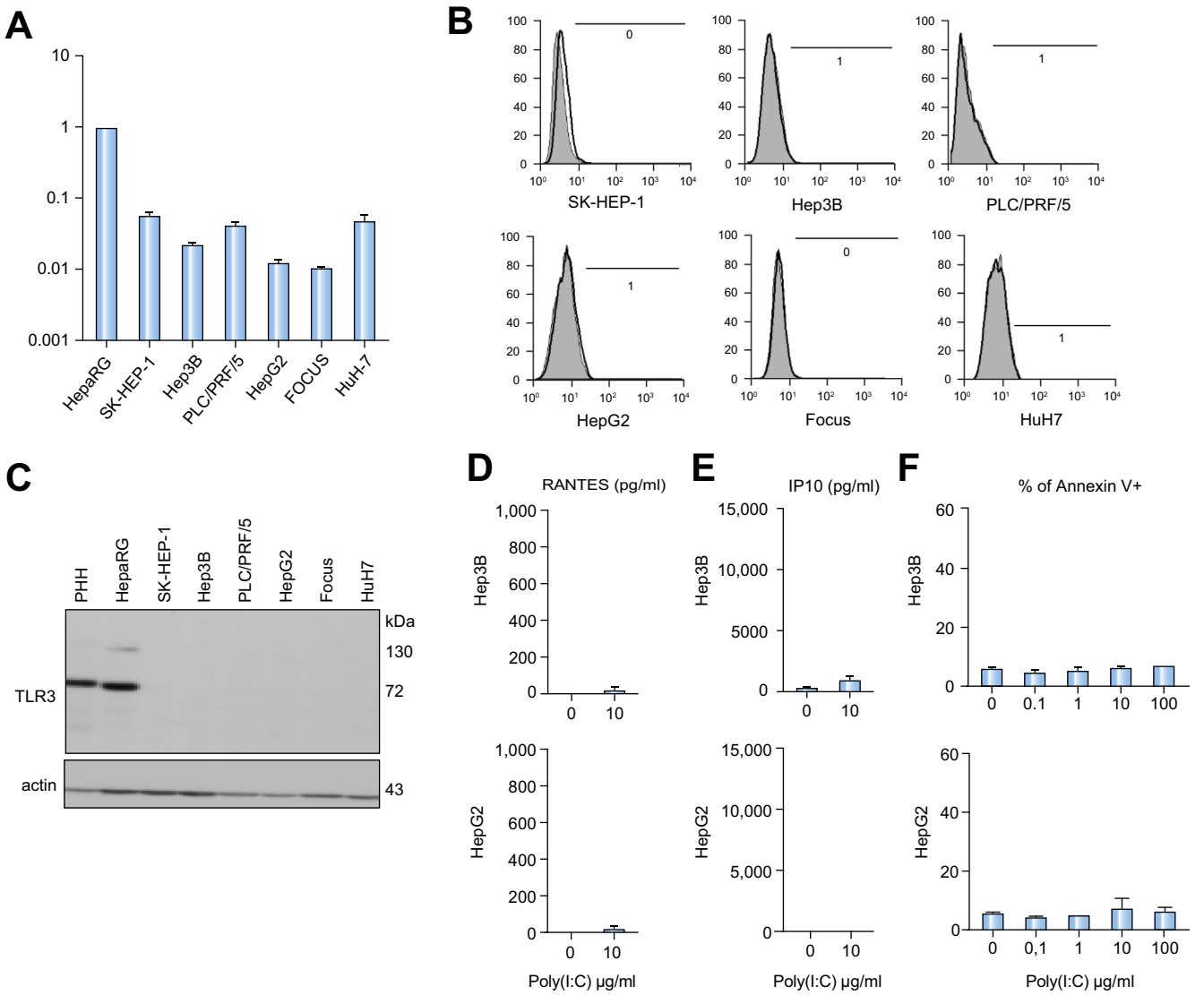

Fig. 3. TLR3 is suppressed in human hepatocarcinoma cell lines. (A) *TLR3* mRNA expression in 6 unstimulated human HCC cell lines compared with HepaRG cells. (B) FACS analysis of intracellular TLR3 expression by 6 human HCC cell lines. Grey = control isotype Ab. Numbers indicate the percentage of $TLR3^+$ cells. (C) Western blot analysis of TLR3 expression by unstimulated PHH and HepaRG cells and in 6 human HCC cell lines. Hep3B and HepG2 cells were treated for 24 h with 10 µg/ml of Poly(I:C) and the concentration of RANTES (D) and IP-10 (E) in the supernatants was measured by ELISA, and the percentage of Annexin V^+ apoptotic Hep3B and HepG2 cells was measured by flow cytometry (F). (A-C) Data are representative of 3 independent experiments. Means ± SEM of 3 independent experiments are shown (D-F). Statistics = two-tailed unpaired t-test (D-F). Ab, antibody; HCC, hepatocellular carcinoma; PHH, primary human hepatocytes.

agreement with the absence of TLR3, Poly(I:C) had no detectable effect on the secretion of RANTES and IP10 (Fig. 3D, E) and on the survival (Fig. 3F) of Hep3B and HepG2 cells, which we then selected for further studies on the functions of TLR3 in transformed hepatocytes.

Re-expression of exogenous TLR3 sensitizes transformed human hepatocytes to TLR3-triggered apoptosis and inflammatory response

To study the functions of TLR3 in HCC cells, we transduced Hep3B and HepG2 cells with either empty (E-LV) or TLR3-HA-encoding (TLR3-LV) doxycycline-inducible lentiviruses, and confirmed the expression of TLR3 by flow cytometry (Fig. 4A) and western blot analysis (Fig. 4B). Of note, in agreement with a previous publication showing that retroviruses can activate TLR3 and IFN-I production,[31] transduction with E-LV or with TLR3-LV without induction was by itself sufficient to drive detectable expression of endogenous TLR3 in approximately 15% of Hep3B cells, corroborating findings by Khvalevsky *et al.*[14] Re-expression of either endogenous TLR3 by E-LV in Hep3B cells and/or exogenous TLR3 in both cell lines had no effect on cell proliferation in standard culture conditions (data not shown), but restored a dose- and cell line-dependent secretion of RANTES and of IP-10 in response

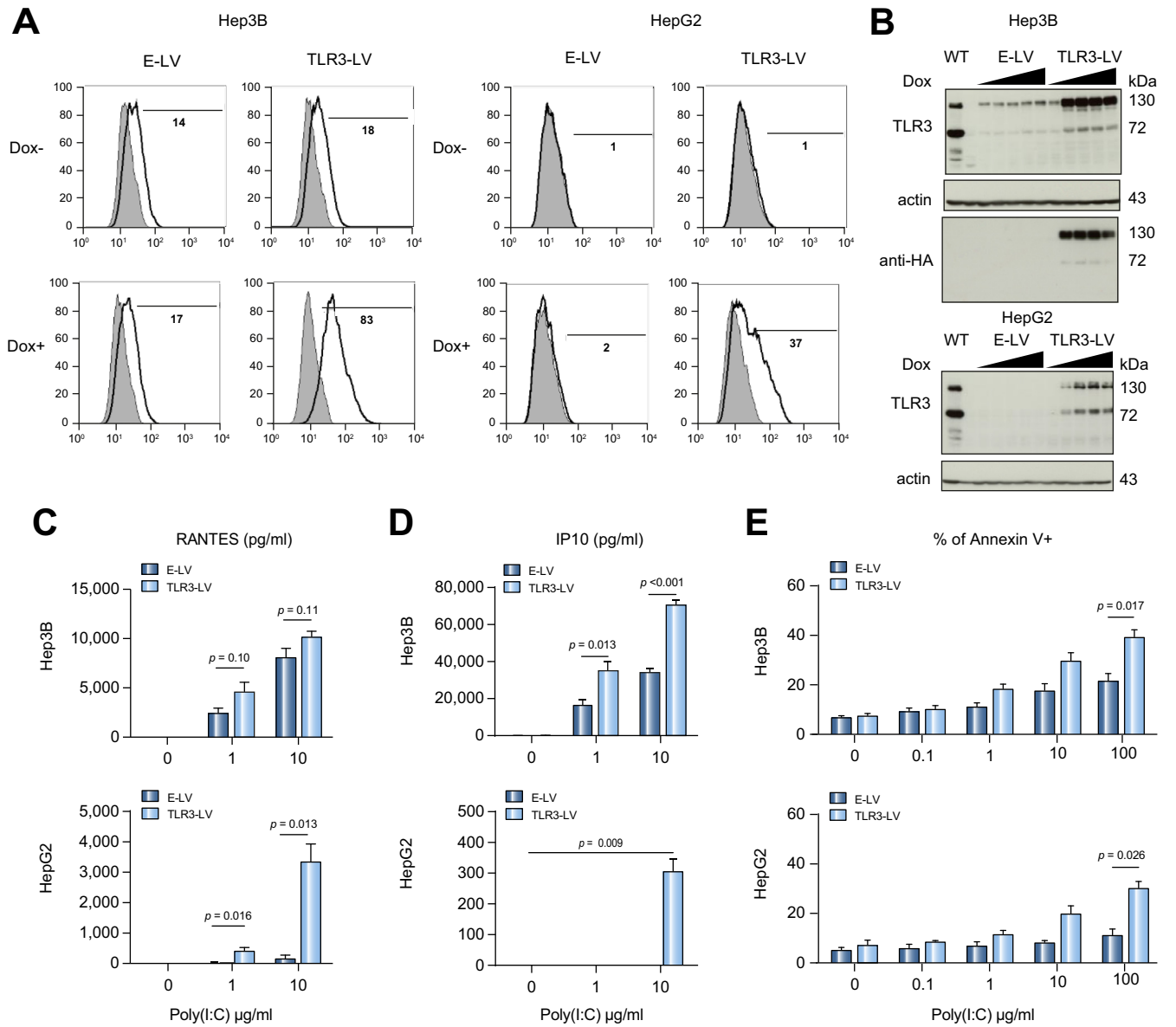

Fig. 4. Re-expression of TLR3 restores inflammatory response and triggers apoptosis in human HCC cell lines. (A) Hep3B and HepG2 cells expressing either empty (E-LV) or TLR3-HA-encoding doxycycline-inducible lentiviral vectors (TLR3-LV) were treated with doxycycline for 24 h and the expression of TLR3 was analyzed by FACS using the anti-TLR3 mAb. Grey = control isotype Ab. Numbers indicate the percentage of TLR3$^+$ cells. (B) The same cells were treated with increasing doses of doxycycline for 24 h and the expression of TLR3 was analyzed by western blot with anti-TLR3 or anti-HA mAb, as indicated. The same cells were treated with doxycycline for 24 h followed by 24 h with increasing doses of Poly(I:C). Secretion of RANTES (C) and IP-10 (D) was measured by ELISA, and the percentage of Annexin V$^+$ apoptotic Hep3B and HepG2 cells was measured by flow cytometry (E). (A, B) Data are representative of 3 independent experiments. Means ± SEM of 3 independent experiments are shown (C-E). Statistics = two-tailed unpaired *t* test (C-E). Ab, antibody; HCC, hepatocellular carcinoma; mAb, monoclonal antibody.

to dsRNA (Fig. 4C, D). Furthermore, and in contrast to WT cells, the forced exogenous (HepG2 cells) and/or endogenous (Hep3B cells) expression of TLR3 in both HCC cell lines resulted in dose-dependent cell death in response to Poly(I:C) (Fig. 4E). This death was an apoptotic cell death, as it was accompanied by caspase 3 activation in the case of HepG2 cells,[14] and Hep3B cells were entirely protected by the pancaspase-inhibitor Z-VAD (Fig. S2).

TLR3 inhibits hepatocarcinogenesis in transgenic mice

As in humans, TLR3 expression was undetectable by western blot in 2 mouse HCC cell lines (LL-11 and MH22a), and was very weak in Hepa1-6 cells compared to normal mouse spleen and liver (Fig. S3A). Moreover, IHC revealed that TLR3 expression was strongly repressed in HCC that developed spontaneously in transgenic AsVB male mice[18] expressing the SV40-TAg in hepatocytes (Fig. 5A), as well as in HCC occurring after treatment of

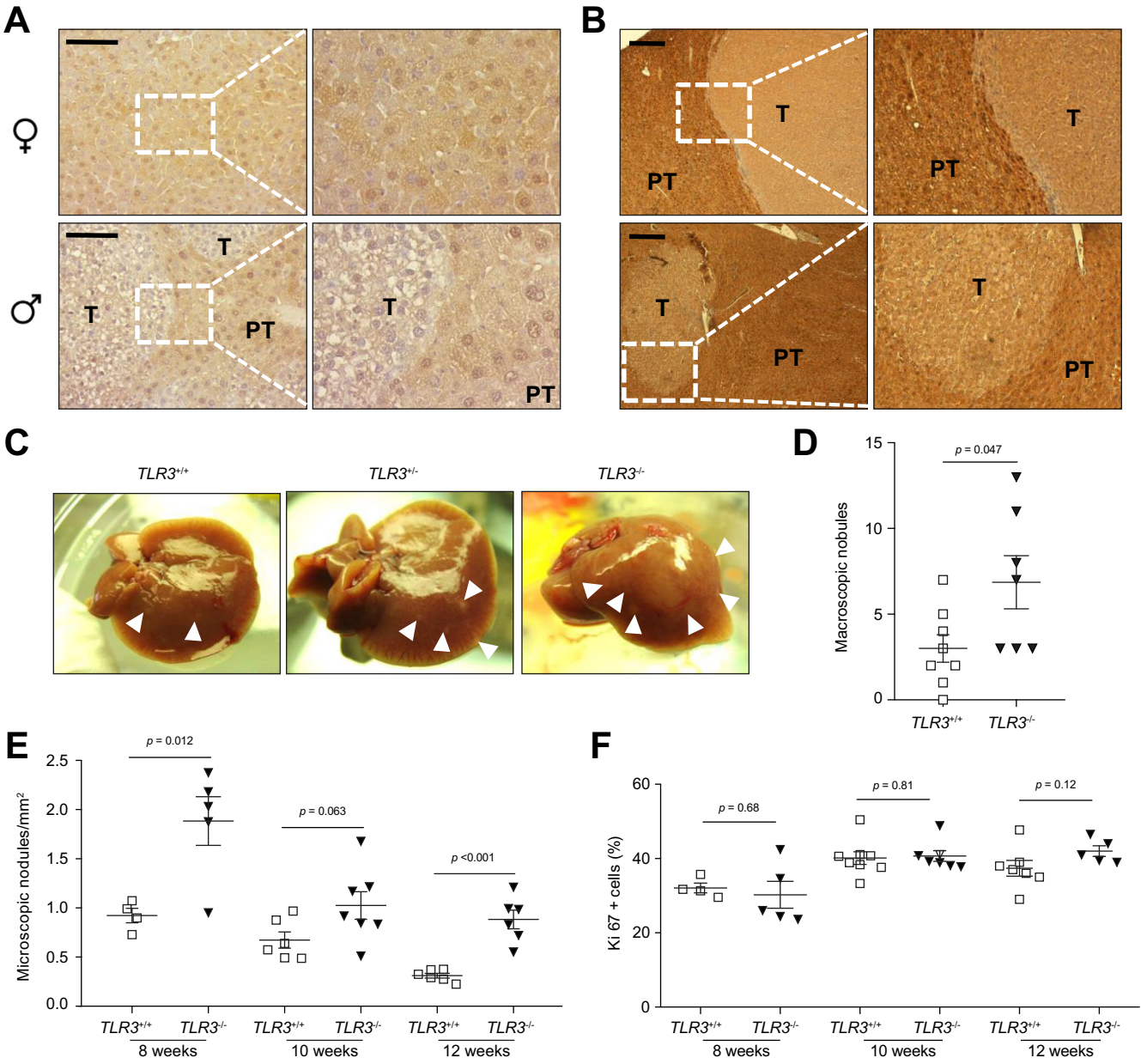

Fig. 5. HCC growth is accelerated in *TLR3*$^{-/-}$ mice. Liver tissue sections from (A) 4-month-old transgenic female and male AsVB mice and from (B) 9-month-old male mice treated with DEN stained with mTLR3-specific antibody. Right images are enlarged views of the white rectangle area in corresponding images on the left (bar = 50 μm). Original magnification = 200×. These images are each representative of >3 livers analyzed. (C) Macroscopic view and (D) number of macroscopic nodules detected at the surface of the livers of 10 weeks-old *TLR3* WT, *TLR3*$^{+/-}$ and *TLR3*$^{-/-}$ AsVB mice. (E) Density of microscopic nodules in livers from 8-, 10- and 12-week-old *TLR3* WT and *TLR3*$^{-/-}$ AsVB mice. (F) Percentage of proliferating Ki67^{+} cells detected by IHC in the liver of 8-, 10- and 12 weeks-old *TLR3* WT and *TLR3*$^{-/-}$ male AsVB mice. Means ± SEM are shown. Statistics = two-tailed Mann-Whitney *U* test (D-F). DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; WT, wild-type. (This figure appears in colour on the web.)

WT mice with DEN (Fig. 5B). To address the role of TLR3 in hepatocarcinogenesis *in vivo*, we crossed AsVB mice with *TLR3* WT and *TLR3*$^{-/-}$ mice and compared the development of liver tumors in their descendants. At 10 weeks of age, the number of macroscopic tumors was higher (two-tailed Mann-Whitney *U* test, $p = 0.047$) in *TLR3*$^{-/-}$ mice than in their *TLR3*$^{+/+}$ littermates (Fig. 5C, D). The number of microscopic tumors was also higher at 8 ($p = 0.012$) and 12 weeks ($p < 0.001$) in *TLR3*$^{-/-}$ than in WT mice (Fig. 5E and Fig. S3B). Strikingly, the average size of tumors was comparable in both groups of mice at 8, 10 and 12 weeks, (Fig. S3C) as was the frequency of Ki67^{+} proliferating cells (Fig. 5F and Fig. 6G). Altogether, these data indicate that the absence of TLR3 was associated with an increased rate of successful hepatocyte transformation without alteration in cancer cell proliferation after their transformation.

Loss of TLR3 is correlated with decreased apoptosis in HCC without alteration of immune cell infiltration

IHC analysis showed no significant decrease in the density of CD3^{+} (Fig. 6A) and CD8^{+} T cells (Fig. 6B) infiltrating tumors in *TLR3*$^{-/-}$ mice compared to *TLR3*$^{+/+}$ mice. Accordingly, analysis by flow cytometry showed no difference in the numbers of CD3^{+} and CD4^{+} T lymphocytes and NK cells infiltrating non-tumoral or tumoral liver from either *TLR3*$^{+/+}$ or *TLR3*$^{-/-}$ mice (Fig. 6C-E). Conversely, the frequency of cleaved caspase 3-positive hepatocytes decreased in the liver of *TLR3*$^{-/-}$ mice

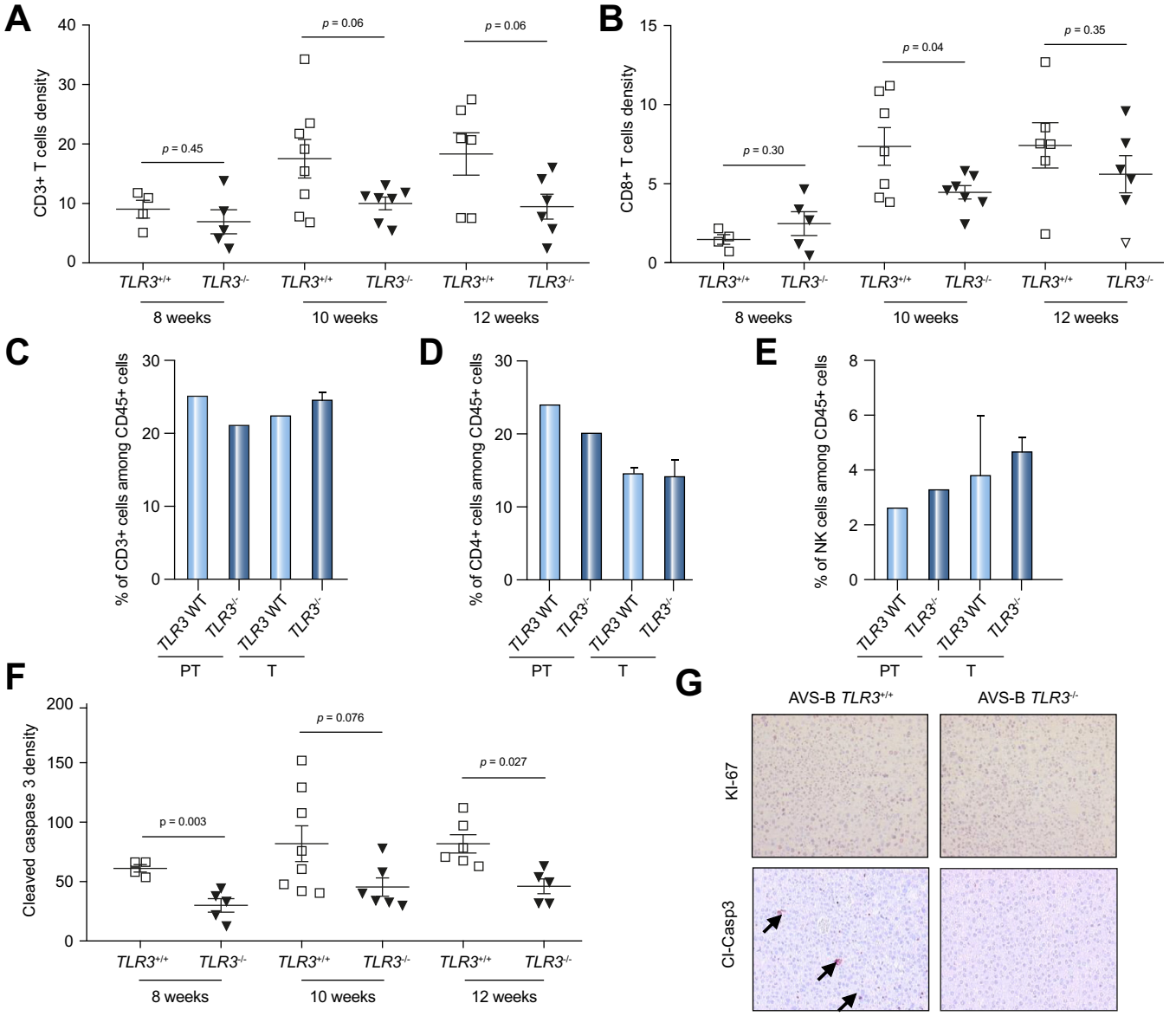

Fig. 6. Absence of TLR3 is associated with decreased apoptosis without alteration of T lymphocyte nor NK cells infiltration in mouse HCC. Density of (A) CD3^{+} T lymphocytes and (B) CD8^{+} T lymphocytes measured by IHC in the livers of 8-, 10- and 12-week-old *TLR3* WT and *TLR3*$^{-/-}$ male AsVB mice. Percentage of CD3^{+} (C), CD4^{+} (D) and NK cells (E) among CD45^{+} cells isolated from peritumoral (PT) and tumoral (T) liver tissues of 12-week-old *TLR3* WT and *TLR3*$^{-/-}$ male AsVB mice (n = 3) measured by flow cytometry. (F) Density of cleaved caspase-3^{+} apoptotic cells measured by IHC in the livers of 8-, 10- and 12-week-old *TLR3* WT and *TLR3*$^{-/-}$ male AsVB mice. (G) Liver tissue sections from 12-month-old WT male AsVB mice stained with anti-Ki67 (upper panel) and anti-cleaved caspase 3 antibody (lower panel). Bar = 50 μm. Original magnification = 100×. Means ± SEM are shown. Statistics = two-tailed unpaired *t* test (A-F). HCC, hepatocellular carcinoma; IHC, immunohistochemistry; NK, natural killer; WT, wild-type. (This figure appears in colour on the web.)

compared to WT mice (Fig. 6F and G), and was inversely correlated with the density of microscopic tumor nodules in male ASV-B mice (Pearson correlation, r = −0.56, $p < 0.001$) (Fig. S4). In summary, in AsVB transgenic mice, TLR3 is downregulated in tumor hepatocytes and hepatocarcinogenesis is accelerated in the absence of TLR3. Consistently, a TLR3 downregulation is accompanied by a reduction in hepatocyte apoptosis without altering liver infiltration of T cells and NK cells.

Discussion

The poor prognosis associated with TLR3 downregulation in patients with HCC has mainly been attributed to the defective recruitment of immune cells and subsequent lack of killing of transformed hepatocytes. The present work demonstrates that TLR3 can also exert an anti-tumoral role in HCC by inducing apoptosis directly in transformed hepatocytes without significantly modulating the tumor microenvironment. We therefore propose that during HCC development, the progressive decrease in TLR3 expression in transformed hepatocytes is an escape mechanism for cancer cells from apoptosis and drives tumor formation. In agreement with previous publications,[20,22] we found that low *TLR3* mRNA expression in human HCC is a predictive factor of HCC recurrence.

The direct apoptotic effect of TLR3 on HCC cells was first established when comparing normal and transformed hepatocytes. We found that TLR3 is expressed and triggers an inflammatory response[31,32] but not apoptosis in normal primary hepatocytes. We further confirmed these results with immortalized, non-transformed HepaRG cells. Published data regarding transformed hepatocytes are more controversial, since human HCC cell lines have been reported either to express TLR3 and respond to Poly(I:C) or not, and except for HepG2[32] and HuH7,[33] the basis for this lack of response was not identified. In the present study, we show that *TLR3* mRNA and protein expression are downregulated in 6/6 human HCC cell lines analyzed, which are not responsive to the TLR3 ligand. Importantly, the forced expression of TLR3 sensitized TLR3-deficient HCC cell lines not only to Poly(I:C)-induced inflammation, but also to apoptosis. Consistently, we have previously shown that TLR3 has a death-receptor function in cancerous bronchial epithelial cells.[34] It is therefore highly pertinent that downregulation of *TLR3* mRNA in patients was not observed during cirrhosis but became detectable once HCC had been diagnosed. Moreover, we demonstrated that downregulation of the TLR3 protein, which we observed in 50% of HCC, exclusively affects transformed hepatocytes but not the normal hepatocytes of the matched peritumoral parenchyma. Altogether, our results suggest that the progressive decrease in TLR3 expression by hepatocytes during their transformation may be a protective mechanism from TLR3-triggered apoptosis, thereby enhancing tumor formation.

Chew *et al.* previously reported that downregulation of TLR3 is included in a 14 immune-gene signature predicting poor patient survival in early HCC. They also proposed a model in which TLR3-dependent apoptosis is mostly indirect, resulting from the killing of HCC cells by cytotoxic immune cells.[15,35] However, after examination of patient HCC samples, we could not confirm the presence of TLR3-positive tumor-infiltrating immune cells by IHC. Furthermore, the expression of TLR3 by human NK cells remains controversial.[36] In addition, we found that the direct TLR3-triggered apoptosis of hepatocytes impaired HCC progression in transgenic AsVB mice independently of NK and T cell infiltration. The apparent discrepancy between our data and the results obtained by Chew *et al.* may be due to the different *in vivo* models used.[36] While Chew *et al.* treated Tg(Alb-lHBV) mice with the synthetic TLR3 ligand Poly(I:C) to activate TLR3 (and MDA5), we did not artificially induce the activation of TLR3 but rather compared spontaneous hepatocarcinogenesis in $TLR3^{+/+}$ with that in $TLR3^{-/-}$ AsVB mice. However, the ligand that triggers TLR3-dependent apoptosis in hepatocytes during their transformation remains unknown. In humans, potential candidates include HCV-derived dsRNA,[6] endogenous retroviruses,[37] LINE's transcripts[38] and/or extracellular dsRNA from intestinal commensal bacteria[39] or released by dying hepatocytes,[40,41] particularly in cells with HBV or HCV. Similar sources of ligands may drive the downregulation of TLR3 in HCC in AsVB mice.

In conclusion, our work provides a set of evidence pointing to a restrictive role for hepatocyte-specific TLR3-triggered apoptosis during hepatocarcinogenesis. It also suggests that the expression of TLR3 in HCC cells may be a useful biomarker to guide clinicians during patient management, in particular to intensify treatments when *TLR3* mRNA is downregulated. Moreover, TLR3 might become a promising target for treating 50% of HCC that do express the receptor.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design, analysis and interpretation of data: Marc Bonnin, Nadim Fares, Philippe Merle and Serge Lebecque; acquisition of data: Marc Bonnin, Nadim Fares, Floriane Pez, Yann Estornes, Lydie Lefrançois, Amandine Garcia, Alain Kfoury, Béatrice Vanbervliet, Isabelle Coste and Baptiste Guey; design and production of lentiviral vectors: Birke Bartoch; immunohistology: Brigitte Bancel and Valérie Hervieu; statistical analysis: Kévin Lang, Alain Viari, Pierre Saintigny, Nadim Fares and Philippe Merle; drafting of the manuscript: Philippe Merle and Serge Lebecque; critical revision of the manuscript for important intellectual content: Yann Estornes, Kathrin Weber, David Durantel, Toufic Renno, Marc Bonnin and Nadim Fares.

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Supplementary data

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Author names in bold designate shared co-first authorship

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