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TLR3 Signaling in a Hepatoma Cell Line is Skewed Towards Apoptosis

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Abstract Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPS) leading to the activation of the innate immune response and subsequently to the shaping of the adaptive immune response. Of the known human TLRs, TLR3, 7, 8, and 9 were shown to recognize nucleic acid ligands. TLR3 signaling is induced by double-stranded (ds)RNA, a molecular signature of viruses, and is mediated by the TRIF (TIR domain-containing adaptor-inducing IFNβ) adaptor molecule. Thus, TLR3 plays an important role in the host response to viral infections. The liver is constantly exposed to a large variety of foreign substances, including pathogens such as HBV (hepatitis B virus) and HCV (hepatitis C virus), which frequently establish persistent liver infections. In this work, we investigated the expression and signaling pathway of TLR3 in different hepatoma cell lines. We show that hepatocyte lineage cells express relatively low levels of TLR3 mRNA. TLR3 signaling in HEK293 cells (human embryonic kidney cells) activated NF-κB and IRF3 (interferon regulatory factor 3) and induced IFNβ (interferon β) promoter expression, which are known to lead to pro-inflammatory cytokine secretion. In Huh7 cells, there was only a short-term IRF3 activation, and a very low level of IFNβ expression. In HepG2 cells on the other hand, while no induction of pro-inflammatory factors was observed, signaling by TLR3 was skewed towards the induction of apoptosis. These results indicate preferential induction of the apoptotic pathway over the cytokine induction pathway by TLR3 signaling in hepatocellular carcinoma cells with potential implications for therapeutic strategies. J. Cell. Biochem. 100: 1301–1312, 2007.

Key words: TRIF; NF-κB; IRF3; Rip1

Mammalian Toll-like receptors (TLRs) serve as an early surveillance against infection by pathogens. To date, 10 TLRs have been identified in humans that recognize conserved pathogen-associated molecular patterns (PAMPs) of microorganisms and viruses. Engagement of TLRs with pathogen components, initiates a signaling cascade in the cell, leading to activation of the innate immune response and shaping of the subsequent adaptive immune response. TLRs are characterized by a ligand-recognizing ectodomain composed of multiple repeats of a leucine-rich motif, and a cytoplasmic signaling domain referred to as a TIR (Toll/IL-I receptor) domain; the TIR domain serves as a platform for assembling multiple protein kinases and adaptor proteins that initiates the signaling process, reviewed in Akira and Takeda [2004].

Among the TLRs, TLR3, 7, 8, and 9, recognize nucleic acids; TLR7 and TLR8 recognize single stranded (ss) RNA [Diebold et al., 2004; Heil et al., 2004] or siRNA (short-interfering RNA) [Hornung et al., 2005]; TLR9 recognizes DNA containing unmethylated CpG motifs [Ahmad-Nejad et al., 2002], and TLR3 recognizes dsRNA derived from viruses or host RNA [Alexopoulou et al., 2001; Kariko et al., 2004]. All four nucleic acid-recognizing TLRs are expressed mainly in the intracellular compartments, whereas all other TLR members are expressed solely on the cell surface [Nishiya and DeFranco, 2004; Nishiya et al., 2005; Kajita et al., 2006].

With the exception of TLR3, all TLR members recruit the adaptor molecule MyD88 (TIR

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domain-containing adaptor molecule myeloid differentiation factor 88), leading to activation of NF-κB and pro-inflammatory cytokine secretion [see review Akira and Takeda, 2004]. TLR3 signaling depends solely on the TIR domain-containing adaptor-inducing IFN-β (TRIF) adaptor protein, which leads to activation of the NF-κB and IRF3 transcription factors, which in turn, induce the expression of IFN-β [Yamamoto et al., 2002, 2003].

The TRIF adaptor protein characteristically induces IFN-β transcription [Yamamoto et al., 2002], a process that requires both, the IRF3 and NF-κB transcription factors [Wathelet et al., 1998]. TRIF-dependent IRF-3 activation is mediated by two non-canonical IKKs, IKKε, and TANK-binding kinase (TBK1) [Fitzgerald et al., 2003; Sharma et al., 2003] which phosphorylate IRF-3. Phosphorylated IRF-3 translocates to the nucleus and induces the expression of numerous host defense genes, including IFNβ [Yang et al., 2004]. The activation of NF-κB by TRIF involves the direct binding of either the TNF receptor-associated factor (TRAF6) protein, or receptor-interacting protein (RIP1), that recruits the IKK complex [Sato et al., 2003; Jiang et al., 2004; Cusson-Hermance et al., 2005] (see model in Fig. 7). Recently, it was shown that in HEK293T cells, TRIF signaling via RIP1 can lead to apoptosis when an additional protein, RIP3, is bound to the RIP1-TRIF complex. When RIP1 and RIP3 bind simultaneously to TRIF, RIP3 inhibits NF-κB induction by RIP1, allowing RIP1 to recruit FADD and to induce the caspase-8-mediated extrinsic apoptotic pathway [Kaiser and Offermann, 2005].

In addition to TLR3, which is located in the endosomal compartment, there are known cytoplasmic proteins, such as PKR, RIG-I, and Mda5, which are also activated by dsRNA and capable of inducing NF-κB and type I IFNs and lead to an antiviral response [Das et al., 1981; Andrejeva et al., 2004; Yoneyama et al., 2004, 2005].

Due to its physiological function, the liver is continuously exposed to a large variety of antigens, including dietary antigens, toxins, and pathogens. Thus, it is perceived that the liver requires specific immunological properties to balance between induction and suppression of the immune response, characterizing the liver immunological environment as an immuno-privileged site. There is strong, albeit indirect evidence, that the type I IFN response is important in the pathogenesis of chronic viral hepatitis. Both hepatitis B virus (HBV) and hepatitis C virus (HCV) have developed mechanisms to disrupt the induction of type I IFNs. Although not well understood, the core protein of HBV inhibits the transcription of IFNβ [Twu and Schloemer, 1989; Whitten et al., 1991], while the NS3/4A protease of HCV was shown to disrupt TRIF and to block TBK1 and IRF3 [Ferreon et al., 2005; Otsuka et al., 2005; Li et al., 2005b]. It thus seems apparent that the TLR3 pathway plays an important role in the outcome of HBV and HCV infection in the liver. We therefore sought to investigate TLR3 signaling in hepatocytes.

Here we report that hepatic cell lineages express relatively low levels of TLR3 mRNA. We observed that TLR3 signaling in the non-hepatic epithelial cell line, HEK293, led to significant activation of NF-κB, and induction of IRF3 and IFNβ expression. However, in both human hepatoma cells, Huh7 and HepG2, TLR3 signaling led to negligible levels of NF-κB induction, but in Huh7 cells, a short-term activation of IRF3 and a low level of IFNβ expression were detected. In HepG2 cells on the other hand, while no TLR3-dependent induction of IRF3 or IFNβ were detected, signaling by TLR3 led to the induction of apoptosis.

MATERIALS AND METHODS

Cells and Reagents

The various cells used in this study are listed in Table I. Cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (Biological Industries, Kibbutz Beit Haemek, Israel), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2% L-glutamine. NK tumor cells (YTS) were grown in RPMI under the same conditions. Peripheral blood mononuclear cells (PBMCs) were purified from blood of healthy donors (received from institutional blood bank as approved by the local IRB) by Hypaque Ficoll density centrifugation. Poly(IC) (Amersham) was used at a final concentration of 50 μg/ml.

Plasmids

Plasmids pTLR7, pTLR8, and pTLR9, carrying the respective human *TLR* gene in the

TABLE I. Cells Used in This Study

Cell name	Origin	Source
HEK293	Human embryonic kidney	ATCC (CRC-1573)
YTS	NK cell tumor	Z. Eshhar (Weizmann Institute of Science, Rehovot, Israel)
T24P	Bladder carcinoma	A. Hochberg (Hebrew University, Jerusalem, Israel)
LS180	Colon adenocarcinoma	ATCC (CL-187)
HepG2		ATCC (HB-8065)
HepG2.2.15		MA. Sells et al. 1987
FLC4		Y. Aoki et al. 1998
FLC4A10		Y. Fellig et al. 2004
Hep3B		ATCC (HB-8064)
Huh7	Hepatoma cell lines	Nakabayashi et al. 1982
PLC/PRF/5		ATCC (CRL-8024)
SNU387		
SNU398		M. Ozturk (Bilkent
SNU449		Univer., Ankara, Turkey)
SNU475		
Normal human hepatocytes	Hepatocytes	Cambrex (Ca. No. CC-2591)

PEF6/V5 vector (Invitrogen) were a gift from TH Chuang (Scripps, La Jolla, CA). Plasmid phTLR3 was constructed by excising the *TLR3* gene from plasmid pUNO-hTLR3 (InvivoGen) and inserting it into plasmid PEF6/V5. To generate phTLR3ΔTIR, the TLR3 TIR domain (amino acids 715–904) (between the BsaBI and SwaI sites) was removed. The reporter plasmids ISRE-luc and p125-luc were a gift from K.A. Fitzgerald, (University of Massacheusetts, Medical School, Worcester, MA). Plasmids pNF-κB-luc and the Renilla-luciferase transfection efficiency vector (pEF-RL) were a gift from Y. Shaul (Weizmann Institute, Rehovot, Israel). pTRIF was a gift from J. Hiscott (Lady Davis Institute, McGill University, Montreal). pCaspase-3-Sensor and pEGFP-N3 (Clontech).

Transfection Assays

For luciferase assays, cells in 24-well plates, were cotransfected using the TransitIT^{R}-LT1 transfection reagent (Mirus), with one of the reporter plasmids: NF-kB-luc (100 ng), ISRE-luc (300 ng), or p125-luc (100 ng); together with 100 ng/ml of a plasmid expressing one of the TLRs, or TRIF, or empty vector; and with 1.5 ng *Renilla reniformis* plasmid (pEF-RL). Each transfection received the same amount of total DNA. We used the Dual-Luciferase Assay System (Promega). For caspase-3 activity assays, cells were seeded on poly-D-lysine treated glass cover slips, and cotransfected with 200 ng pCaspase-3-Sensor plasmid, together with 100 ng phTLR3 or empty vector. For cell-cycle experiments, cells seeded in 3-cm plates, were cotransfected with 3 μg pEGFP-N3, together with 2 μg phTLR3 or pTRIF, or empty vector.

RNA Quantification

RNA was isolated using TRI-Reagent (Sigma), followed by DNaseI treatment using the DNaseI Kit (Ambion). Quantitative real-time PCR (qRT-PCR) assays were performed using the ABI PRISM 7700 Sequence Detector system (Applied Biosystems). Primers/probe sets were obtained from Applied Biosystems: Hs00152933_m1 and Hs01551078_m1 for TLR3 (recognizing exones 1–2 and 3–4, respectively); Hs00152971_m1 for TLR7; Hs00152873_m1, and Hs00370913_s1 for TLR9 (recognizing splice variants A and B, respectively); and Hs99999902_m1 to detect ribosomal protein large subunit P0 (*RPLP0*) gene used as an endogenous control. Threshold cycle numbers (Ct) were determined with Sequence Detector Software (version 1.6; Applied Biosystems) and transformed using the ΔCt method as described by the manufacturer.

Immuno-Staining and Flow Cytometry

For cell-cycle analyses, cells were harvested and fixed in 70% ethanol, washed and re-hydrated in PBS for 30 min. The cells were then resuspended in PBS containing propidium iodide (5 μg/ml) and RNase A (50 μg/ml), and kept at 4°C for 1 h before analyses. Analysis were performed on a FACS Calibure flow cytometer (Becton Dickinson, San Jose, CA) using the Cell Quest software.

RESULTS

Expression of TLR3, TLR7, and TLR9-Specific RNA in Hepatic and Non-Hepatic Cells

TLR3 is known to be expressed in human Dendritic cells, NK cells, and was also detected in non-lymphoid tissues such as placenta, pancreas, lung, heart, brain, and liver [Cario and Podolsky, 2000; Muzio et al., 2000; Matsumoto et al., 2003; Schmidt et al., 2004; Nishimura and Naito, 2005]. In lymphoid and epithelial cells, TLR3 signaling leads to NF-κB and IRF3 activation and eventually to secretion of inflammatory cytokines and type I interferons [Alexopoulou et al., 2001; Hardy et al., 2004; Matsushima et al., 2004]. In HEK293 cells, TLR3 mRNA levels were reported to be low but still sufficient to induce minimal type I IFN production [Kariko et al., 2004].

In the current study we explored the function of TLR3 in hepatocyte lineage cells. First, we assessed the level of TLR3 mRNA in different hepatic and non-hepatic cells (see Table I) using relative quantitative real-time PCR (qRT-PCR) analysis, performed on total RNA extracted from the various cells. The level of TLR3 mRNA in each cell type was normalized to that of HEK293 cells. As seen in Figure 1A (left panel) in most hepatic lineage cells, TLR3 mRNA levels were low, compared to HEK293, while the mRNA level in HEK293 was sixfold lower than its level in peripheral blood mononuclear cells (PBMCs). Normal human hepatocytes expressed high TLR3 mRNA levels, similar to PBMCs and LS180 (colon adenocarcinoma).

In parallel to TLR3, we assessed the mRNA levels of two additional nucleic acid-recognizing TLRs, TLR7, and TLR9, using the same method described above. Endogenous TLR9 mRNA persists in two isoforms: the longer one, isoform *a*, and a shorter one, isoform *b*. The results shown in Figure 1A (middle panel) reveal that except for Huh7 cells which expressed TLR7 mRNA levels similar to HEK293 cells, the rest of the hepatic cell lineages expressed significantly lower levels of TLR7 mRNA. TLR9 mRNA levels (Fig. 1A right panel) were not much different than that of HEK293. PBMCs expressed high TLR7 and 9 mRNAs levels.

A comparison of the mRNA level of the various TLRs within HEK293 revealed that TLR3, TLR7, and TLR9b were expressed at similar levels, whereas TLR9a was significantly lower (data not shown). Taken together, the results demonstrate that overall, hepatoma cell lines express low levels of TLR 3, 7, and 9 mRNAs.

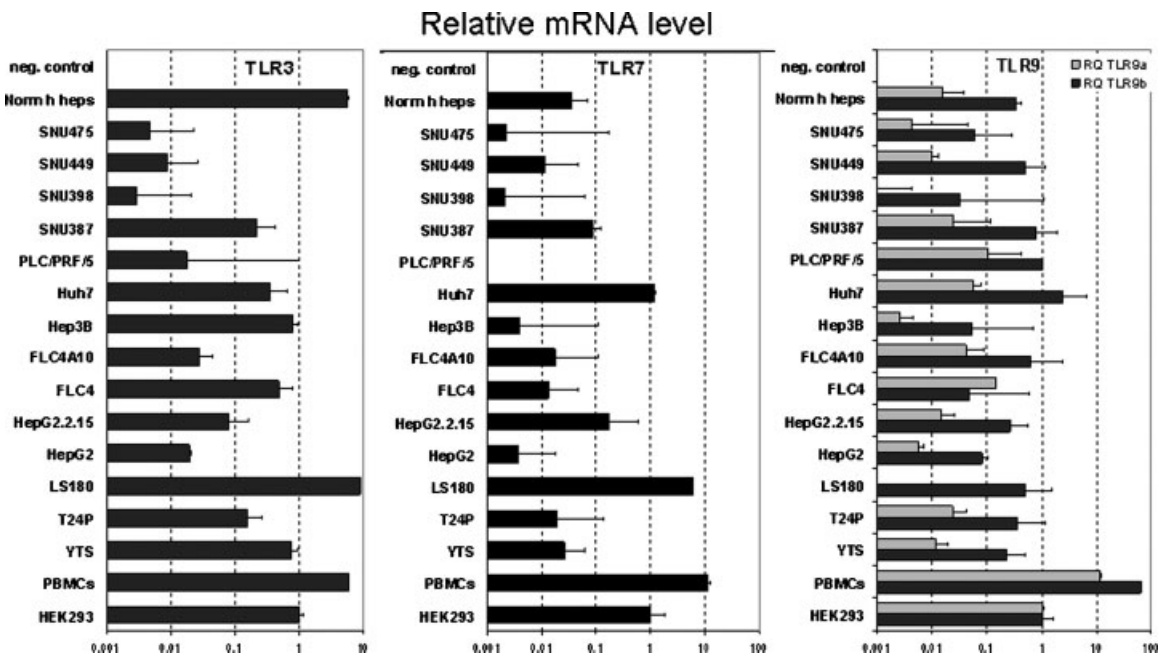

Fig. 1. Relative quantitative real-time PCR (qRT-PCR) of TLR3, TLR7, and TLR9 mRNA. Shown are the mRNA levels of the different TLRs relative to their level in HEK293 cells. The right panel shows relative mRNA levels of the two splice variants of TLR9, *a* and *b*.

TLR3 Signaling in HepG2, Huh7, and HEK293 Cell Lines

We were interested in exploring TLR3 signaling in hepatocytes. We used the human hepatocellular carcinoma cell lines HepG2 and Huh7. Both cell lines have similar gene expression profile with upregulated α-fetoprotein [Lee and Thorgeirsson, 2002], but they differ in several pathways related to apoptosis, such as the Wnt-β-catenin pathway which is impaired in HepG2 cells, and the p53 pathway which is impaired in Huh7 cells [Erdal et al., 2005].

In order to explore TLR3 signaling, we transiently transfected HEK293, Huh7, and HepG2 cells with either, a TLR3-bearing plasmid (phTLR3), a plasmid carrying a truncated form of TLR3 lacking the TIR domain (phTLR3ΔTIR) or an empty vector. TLR3 mRNA expression levels were determined by qRT-PCR. As seen in Figure 2, all transfected cell lines expressed high levels of plasmid-born TLR3 and TLR3ΔTIR mRNAs.

TLR3 signaling is known to lead to the activation of the NF-κB and IRF3 transcription factors which in turn, induce the expression of IFNβ [Takeda and Akira, 2004]. We compared TLR3-induced NF-κB activation in hepatic cell lines to that in HEK293 cells, using the NF-κB-luc reporter plasmid. Briefly, cells were co-transfected either with phTLR3 or empty vector, together with the NF-κB-luc reporter plasmid, and a Renilla expressing plasmid. To demonstrate the specificity of the TLR3 response, the cells were transfected in parallel with the phTLR3ΔTIR plasmid expressing the truncated *TLR3* gene. TLR3 signaling was induced by the addition of poly(IC) to the culture medium. The results shown in Figure 3A (left panel), reveal that only low levels of NF-κB were induced in HepG2 and Huh7 cells upon TLR3 activation, as compared to HEK293 cells. The same results were obtained when poly(IC) was introduced into the cells with a transfection reagent (data not shown). It should be noted that a certain degree of NF-κB activation was obtained in all the cells, solely from the overexpression of TLR3.

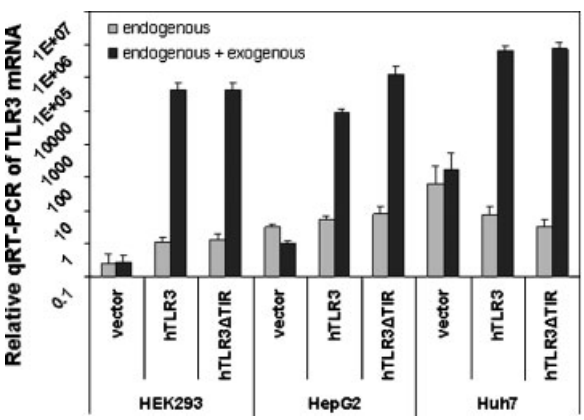

Fig. 2. Endogenous and plasmid-born TLR3 mRNA levels. HEK293, HepG2, and Huh7 cells were transfected with phTLR3, phTLR3ΔTIR, or with empty vector plasmids. cDNA was prepared from total RNA extracted 48 h post-transfection, and qRT-PCR was performed using two sets of primers and probes; the first set that recognizes only endogenous TLR3 mRNA (endogenous), and a second set that recognizes both, endogenous and exogenous TLR3 mRNA (endogenous + exogenous), including that of TLR3ΔTIR. The graph presents mRNA levels calculated relative to the mRNA level of TLR3 in HEK293 cells transfected with empty vector, as determined by the second primer set (endogenous + exogenous).

Next, we tested IRF3 activation and IFNβ promoter induction. To assess IRF3 activation we used the ISRE-luc reporter plasmid, which contains the IRF3 binding site taken from the regulatory region of the *IFNβ* gene [Harada et al., 1989]. Induction of the IFNβ promoter was assessed by the use of the p125-luc reporter plasmid [Fujita et al., 1993]. The results show that in HepG2 cells, no TLR3-dependent induction of ISRE or p125 was observed at all time points (Fig. 3A, middle and right panels and Fig. 3B). In Huh7 cells, TLR3 activation led to a low level of p125 induction compared to HEK293 cells, whereas, ISRE induction was significant, and reached a level comparable to that in HEK293 cells, already at 6 h after induction. However, while in HEK293 cells ISRE induction was continuous, in Huh7 cells it declined by 24 h (Fig. 3A,B) and was not detected at the 36-h time point (data not shown). The effect of TLR3ΔTIR expression, in all the three lines, was similar to the effect of empty vector.

A low level of poly(IC) induction of ISRE and p125, which was independent of the plasmid-born TLR3, was observed in the HepG2 and HEK293 cell lines, but not in Huh7. This could be due to the induction of endogenous TLR3, and/or to the induction of an alternative, TLR3-independent pathway, such as RIG-I, which was shown to be induced by poly(IC) [Yoneyama et al., 2004].

Part of the TRIF and the MyD88-mediated pathways induced by TLRs, converge at the stage of the IκB kinases, IKKα, and IKKβ, which

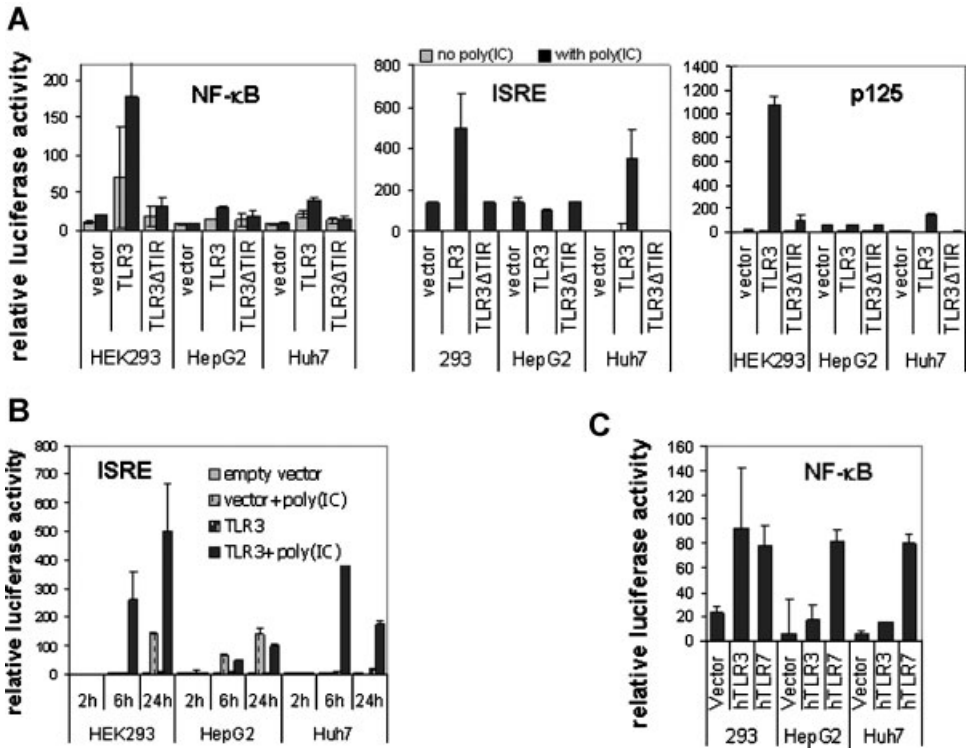

Fig. 3. TLR3-mediated activation of NF-κB, IRF3, and IFNβ. **A**: Luciferase reporter plasmids for NF-κB, IRF3 (ISRE), and IFNβ (p125), were transfected into HEK293, HepG2, and Huh7 cells, together with plasmids expressing either hTLR3, hTLR3ΔTIR, or empty vector, and a Renilla expressing plasmid. The next day, poly(IC) (50 μg/ml) was added to the culture medium and the cells were harvested 18 h later. Shown are the ratios of luciferase versus Renilla activities. **B**: Cells were transfected with a TLR3 expressing plasmid or empty vector, together with the ISRE-luc reporter plasmid and a Renilla expressing plasmid. TLR3 signaling was induced the following day by the addition of poly(IC) (50 μg/ml). Luciferase versus Renilla activities were determined at 2, 6, and 24 h after the addition of poly(IC). **C**: Cells were transfected with either phTLR3, phTLR7, or empty vector, together with the NF-κB-luc reporter plasmid and Renilla expressing plasmid. Shown are the ratios of luciferase versus Renilla activities. The graphs show representative results of at least three independent experiments.

directly phosphorylate members of the inhibitory IκB family, leading to NF-κB activation. To assess the functional integrity of the shared segment of the pathway in the hepatoma cell lines, we tested NF-κB activation by overexpression of TLR7, which signals via the MyD88 adaptor. As seen in Figure 3C, contrary to TLR3, TLR7 overexpression induced NF-κB in the hepatoma cell lines to approximately similar levels as HEK293, indicating that the shared TRIF-MyD88 NF-κB activation pathway, is not impaired in these cell lines.

The Effect of TRIF Overexpression on IRF3 Induction

TLR3 signaling is mediated by the TRIF adaptor protein [Yamamoto et al., 2002]. Overexpression of TRIF in HEK293 cells has previously been shown to induce the TLR3 signaling pathway, as measured by IRF3 activation [Yamamoto et al., 2002; Oshiumi et al., 2003]. To test whether overexpression of TRIF in hepatic cell lines mimics the effect of TLR3 induction, cells were co-transfected with a TRIF-expressing plasmid together with the ISRE-luc reporter plasmid, and the Renilla-expressing plasmid. At different time points after transfection, relative luciferase activity was determined. Figure 4 reveals that TRIF overexpression resulted in a significant induction of IRF3 in HEK293 cells, which increased for at least 30 h post-transfection. In contrast, in HepG2 and Huh7 cells, only a negligible level of induction was observed at all time points.

To study the effect of TRIF overexpression on the induction of NF-κB, we performed similar experiments to those described above, using the NF-κB-luc reporter plasmid. We found that overexpression of TRIF did not induce NF-κB activity in all three cell lines (data not shown).

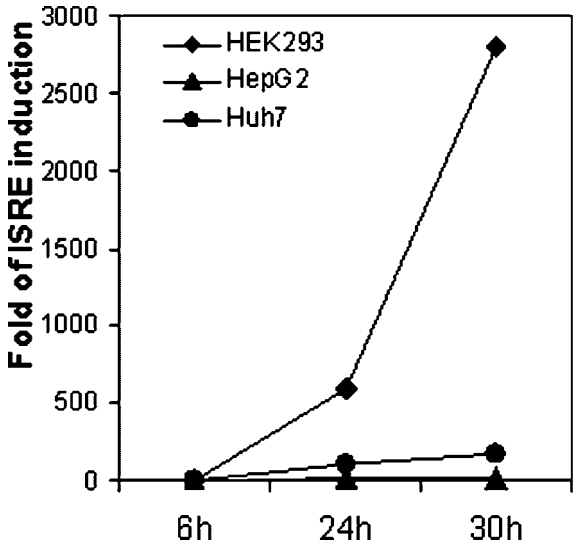

Fig. 4. The effect of TRIF overexpression on IRF3 activation. The indicated cell lines were co-transfected with a TRIF expressing plasmid or with empty vector, together with the ISRE-luc reporter plasmid, and a Renilla expressing plasmid. Relative luciferase activity (luciferase/renilla activity ratio) was determined at 6, 24, and 30 h after transfection. The results represent data from three independent experiments.

TLR3 Signaling Leads to Apoptosis in HepG2 Cells

It has recently been reported that TLR3 activation may lead, on the one hand, to the induction of NF-κB and IRF3, and on the other hand, to apoptosis via RIP1 and caspase-8 activation [Schroder and Bowie, 2005]. TRIF overexpression alone, may also lead to apoptosis via the same pathway [Kaiser and Offermann, 2005]. To test whether TLR3 induction leads to apoptosis in hepatoma cells, we co-transfected cells with either a TLR3 expressing plasmid or with an empty vector, together with an EGFP expressing plasmid. The next day, TLR3 signaling was triggered by the addition of poly(IC), and the cells were harvested 4, 6, and 15 h thereafter. Cell-cycle analysis were performed by PI staining and flow cytometry on GFP-positive cells, and the percentage of cells in the sub-G_1 fraction at the different time points was calculated. The results (Fig. 5A) demonstrate that poly(IC) led to a significant TLR3-dependent increase in the number of HepG2 cells in the sub-G_1 fraction, reaching 70% by 15 h. At the 24-h time point, poly(IC) induced high levels of apoptosis also in cells transfected with empty vector, possibly due to the induction of endogenous TLR3. In contrast to HepG2 cells, in HEK293 and Huh7 cells, TLR3 had no effect on the number of cells in the sub-G_1 frasction.

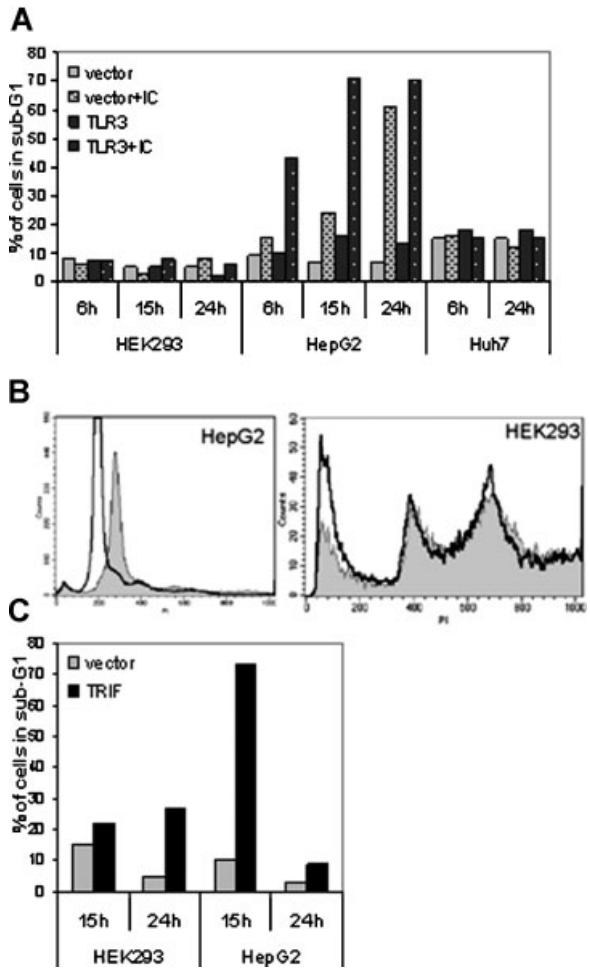

Fig. 5. Activation of TLR3 leads to apoptosis in HepG2 cells. Cells were transfected with a TLR3-expressing-plasmid, a TRIF-expressing plasmid or with empty vector, together with an EGFP-expressing plasmid. **A**: TLR3 signaling was induced 1 day after transfection, by the addition of poly(IC) (50 μg/ml). Cell-cycle analysis was performed by flow cytometry on PI stained cells, harvested at the indicated time points after the addition of poly(IC). For each sample, 10^4 EGFP-expressing cells were measured. **B**: Shown is a representative flow cytometric analyses at the 15-h time point. Black line represents TRIF transfected cells; filled gray areas represent transfection with empty vector. **C**: The graph shows the percentage of cells in the sub-G_1 phase at the different time points following transfection with TRIF-expressing plasmid (TRIF) or empty vector (vector).

To verify that the TLR3-signaling pathway was responsible for the induction of apoptosis, we overexpressed TRIF in the three cell lines and performed cell-cycle analysis 15 and 24 h after transfection. Figure 5B shows representative data of cell-cycle analysis in HepG2 and

HEK293 cells, 15 h after transfection. The percentages of cells in the sub-G_1 fraction at the different time points are summarized in Figure 5C. The results reveal that TRIF overexpression in HEK293 cells, in accordance with previous reports [Kaiser and Offermann, 2005], increased only slightly the percentage of cells in the sub-G_1 fraction compared to empty vector. In HepG2 cells, on the other hand, TRIF overexpression led to a significant increase in the apoptotic cell fraction 15 h after transfection, reaching approximately 70% of the transfected cells. In correlation with the increase in the sub-G_1 fraction, a decrease in the number of cells at G_1 was observed (data not shown). At the 24-h time point, the percentage of apoptotic HepG2 cells declined to 9% of the EGFP-expressing cells, which was still threefold higher than that of cells transfectd with empty vector. This decrease in the sub-G_1 fraction is most probably due to the death of the apoptotic cells.

To further demonstrate that in HepG2 cells TLR3 signaling is skewed towards the induction of apoptosis, we used the Caspase-3-Sensor reporter plasmid carrying a Caspase-3-EYFP fusion protein with a nuclear export signal (NES) at the N-terminus, followed by a caspase-3 cleavage site, and a nuclear localization signal (NLS) at the C-terminus. In non-apoptotic cells, the encoded Caspase-3-EYFP fusion protein resides in the cytoplasm. Upon induction of apoptosis, activated caspase-3 cleaves off the NES domain and the protein translocates to the nucleus. To test whether TLR3 signaling in HepG2 cells leads to activation of caspase-3, we cotransfected cells with either phTLR3 or with empty vector, together with the Caspase-3-Sensor plasmid. Two days after transfection, poly(IC) was added to the medium, and the percentage of cells carrying EYFP in the nucleus versus the total number of transfected cells, was determined at 1, 3, and 6 h after the addition of poly(IC). Figure 6A shows an example of a HepG2 cell before poly(IC) induction, where EYFP is present in the cytoplasm (left image), and a cell after induction,

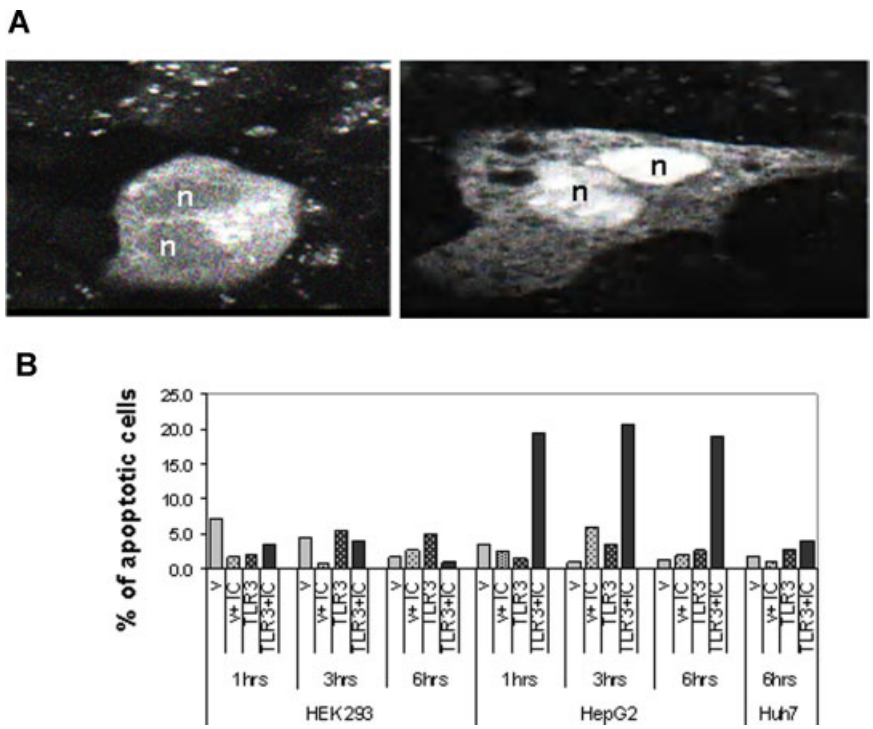

Fig. 6. Activation of caspase-3 by TLR3 signaling. Cells were transfected with a TLR3-expressing-plasmid, or with empty vector, together with the Caspase-3-Sensor plasmid. TLR3 signaling was induced by the addition of poly(IC) (50 μg/ml) 1 day after transfection. **A**: Representative HepG2 cells before induction (left panel) where EYFP (white color) is distributed in the cytoplasm, and 1 h after poly(IC) induction (right panel), where most of the EYFP is found in the nucleus (n). **B**: At the indicated time points after poly(IC) addition, cells were fixed and EYFP expression and cellular location were determined by fluorescence microscopy. For every treatment, at least 200 transfected cells were analyzed. The graph represents the percentage of cells expressing EYFP in the nucleus relative to the total number of EYFP expressing cells.

where most of the EYFP has translocated to the nucleus (right image). The results summarized in Figure 6B reveal that in HEK293, no TLR3-dependent induction of caspase-3 activity was observed. Furthermore, the number of transfected cells was stably maintained for the duration of the experiment (data not shown). In HepG2 cells, on the other hand, within 1 h of poly(IC) induction, approximately 20% of the TLR3-transfected cells contained EYFP in the nucleus, compared to 4% of cells transfected with empty vector. Three and 6 h after poly(IC) induction, the percentage of cells with EYFP in the nucleus remained at the same level. It is important to note, however, that the total number of HepG2 cells transfected with TLR3 and induced by poly(IC) declined to 50% compared to non-induced or to empty vector-transfected cells, at the 3- and 6-h time points (data not shown). This decline could be due to the induction of cell death following the activation of TLR3 signaling. In Huh7 cells, in agreement with the results of the cell-cycle analysis, no TLR3-dependent induction of apoptosis was observed.

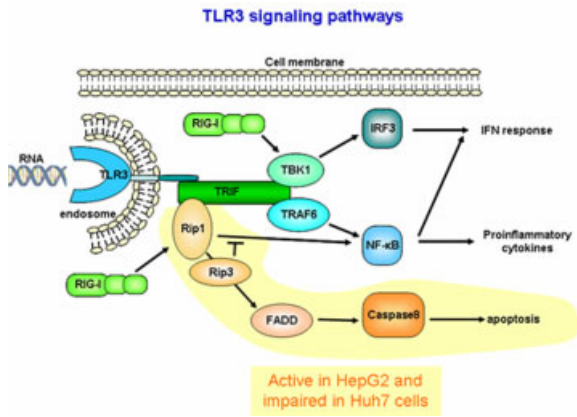

Fig. 7. TLR3-signaling pathway: TLR3 is localized in the endosomal compartment. Upon ligand binding, TRIF is recruited, and induces signaling pathways, leading to induction of NF-κB and IRF3 and the initiation of the apoptotic cascade. IRF3 induction is mediated by TBK1. NF-κB can be induced by RIP1 or TRAF6. RIP3 blocks NF-κB activation by RIP1 and enables its binding to FADD, consequently leading to caspase-8 activation and apoptosis. RIG-I can induce NF-κB via RIP1 and IRF3 via TBK1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

In the present study we show that hepatic cells express low levels of TLR3. Furthermore, we show that in the hepatoma cell lines, HepG2 and Huh7, activation of the TLR3-signaling pathway via the TRIF adaptor protein, did not lead to the activation of the key cytokine-inducing transcription factors, NF-κB or IRF3. Rather, in HepG2 cells, TLR3 signaling resulted in the induction of apoptosis (a schematic presentation of the TLR3-signaling pathway is shown in Fig. 7).

In agreement with a previous report [Doyle et al., 2002], overexpression of TLR3 in HEK293 cells followed by the addition of poly(IC), activated NF-κB and IRF3 and induced IFNβ promoter activity. This induction was mediated by the TIR domain, since overexpression of phTLR3ΔTIR had only a negligible effect (Fig. 3A). Overexpression of TLR3 without poly(IC) also resulted in a lower, but significant, induction of NF-κB. The response of HepG2 and Huh7 to TLR3 activation, differed from that of HEK293 in several ways. First, since no noteworthy levels of NF-κB activity were induced in both cell lines, we suggested that in these cells, the TLR3 pathway leading to NF-κB induction is not functional. However, we showed that TLR7 overexpression (Fig. 3C) as well as TLR8 (data not shown) resulted in a notable level of NF-κB induction in all three cell lines, HEK293, HepG2, and Huh7. This indicates that at least the TRIF-MyD88 mutual segment of the NF-κB pathway, beginning at the phosphorylation of the IKKα and IKKβ, is not impaired in both hepatoma cell lines. Second, IRF3 induction was observed in HEK293 but not in HepG2 cells, and only transiently in Huh7 cells (Fig. 3B).

The finding that TLR3 signaling differs between HEK293 and the hepatic lineage cells, gains support from our experiments with the TLR3 adaptor molecule, TRIF. TRIF overexpression induced a robust IRF3 induction in HEK293, no induction in HepG2, and a very low level of induction in Huh7 cells. The absence of TLR3-dependent IRF3 induction in HepG2 was previously reported by Li et al. [2005a]. But contrary to our results, they found the same effect in Huh7 cells as well. However, they do note that there are differences between Huh7 cell lines propagated among different laboratories.

TLRs provide the first line of defense against invading pathogens and are capable of directing the infected cells either towards the secretion of pro-inflammatory cytokines or to its elimination

by apoptosis. It was previously shown that the TLR3-TRIF signaling pathway can induce apoptosis through the activation of the FADD-caspase-8 axis [Kaiser and Offermann, 2005]. Our experiments demonstrated that in Huh7 cells, no TLR3-dependent apoptosis occurs, while in HepG2 cells both, TLR3 signaling and TRIF overexpression, induced apoptosis.

In Huh7 cells, TLR3 signaling did not induce NF-κB nor did it induce apoptosis, both of which require the activity of RIP1 [Kelliher et al., 1998; Meylan et al., 2004; Kaiser and Offermann, 2005]. We therefore hypothesize that RIP1 activity is impaired in our Huh7 cell line. RIP1 was also shown to be required for the induction of NF-κB by RIG-I [Kawai et al., 2005]. Li et al. [2005a] have shown that in their Huh7 cell line, both, the TLR3 and the RIG-I pathways, were impaired. Since RIP1 is shared by both pathways, it is consistent with our hypothesis of RIP1 impairment in our Huh7 cells, which could explain why TLR3 activation did not lead to apoptosis.

In HepG2 cells, TLR3 signaling did not induce NF-κB nor IRF3; rather, it led to the induction of apoptosis. TLR3-dependent induction of NF-κB is predominantly mediated by RIP1 and also by TRAF6 [Jiang et al., 2004; Meylan et al., 2004], while IRF3 induction requires TBK1. Our results suggest that TBK1, TRAF6, and RIP1 are functional in HepG2 for the following reasons; (i) TLR3-independent poly(IC) induction of IRF3 (Fig. 3A,B) shown to be mediated by TBK1 [Fitzgerald et al., 2003] seems to be active; (ii) TRAF6 is required for NF-κB induction in the MyD88-mediated pathway and is probably functional since TLR7 overexpression resulted in NF-κB induction (Fig. 3C); and (iii) RIP1 is pivotal for TRIF-mediated apoptosis and therefore we assume it is functional in HepG2 cells. Thus, considering the factors known today, TLR3 signaling in HepG2 cells is not impaired.

We hypothesize that if TLR3 signals in the same fashion in normal hepatocytes, namely by induction of apoptosis, with no induction of the inflammatory response, it may contribute to the establishment of the immune privileged state of the liver, a state where the immune response is suppressed. However, the role of TLR3 signaling in normal hepatocytes requires further investigation in vivo.

Recently, it was shown that TLR3 can directly trigger apoptosis upon poly(IC) induction in some human breast tumor cells [Salaun et al., 2006], similar to our finding with HepG2 cells. The ability of TLR3 to induce apoptosis in tumor cells opens a new avenue for therapeutic applications using TLR3 agonists for the treatment of selected cancers.

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