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Expression and Functionality of Toll- and RIG-like receptors in HepaRG Cells

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Primary human hepatocyte (PHH) ; toll-like receptor (TLR) ; retinoic acid-inducible gene 1 (RIG-I) ; RIG-like receptor (RLR) ; reverse transcription (RT) ; quantitative polymerase chain reaction (qPCR) ; fluorescence-activated cell sorting (FACS) ; interleukin-6 (IL-6) ; interferon-gamma-inducible protein-10 (IP-10) ; interferon (IFN) ; hepatitis B virus (HBV) ; melanoma differentiation-associated gene 5 (MDA-5) ; Hepatitis A virus (HAV) ; hepatitis E virus (HEV) ; hepatitis D virus (HDV) ; hepatitis C ; world health organization (WHO) ; hepatocellular carcinoma (HCC) ; pathogen recognition receptor (PRR) ; C-type lectin receptor CLR) ; NOD-like receptor (NLR) ; deoxy-ribonucleic acid (DNA) ; pathogen-associated molecular pattern (PAMP) ; double-stranded ribonucleic acid (dsRNA) ; sodium taurocholate cotransporting polypeptide (NTCP) ; differentiated HepaRG (dHepaRG) ; american type culture collection (ATCC) ; dimethysulfoxide (DMSO) ; complementary DNA (cDNA) ; cycle threshold (CT) ; lipoteichoic acid (LTA), lipopolyipopolysaccharide (LPS) ; oligonucleotie (ODN) ; low molecular weight (LMW) ; Ethylenediaminetetraacetic acid (EDTA) ; sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) ; hepatitis B « e » antigen (HBeAg) ; hepatitis B « s » antigen (HBsAg) ; enzyme-linked immunosorbent assay (ELISA) ; proliferative HepaRG (pHepaRG) ; nuclear factorkappa B (NF-κB) ;

Key Words: pathogen recognition receptor (PRR), toll-like receptor (TLR), hepatocyte, hepatitis B virus (HBV), antiviral activity

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Authors contribution: SL, MAG, MM, OF, MG, MB, JL, DD performed experiments; SL**,** MAG, SF, HJ, FZ, DD designed experiments; SL, MAG, MM, OF, MB, JL, DD analysed results and drawn figures; SL, MAG, FZ, DD wrote the manuscript.

**contributed equally, ¶contributed equally*

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Abstract

Background & Aims: HepaRG cells are considered as the best surrogate model to primary human hepatocyte (PHH) culture to investigate host-pathogen interactions. Yet their innate immune functions remain unknown. In this study, we studied the expression and functionality of Toll-like (TLR) and retinoic-acid-inducible gene-1 (RIG-I)-like (RLR) receptors in these cells.

Methods: Gene and protein expression levels of TLR-1 to 9 and RLR in HepaRG were mainly compared to PHH, by RT-qPCR, FACS, and Western Blotting. Their functionality was assessed, by measuring the induction of *toll/rig-like* themselves and several target innate gene expressions, as well as the secretion of IL-6, IP-10, and type-I interferon (IFN), upon agonist stimulation. Their functionality was also evidenced, by measuring the antiviral activity of some TLR/RLR agonists against hepatitis B virus (HBV) infection.

Results: The basal gene and protein expression profile of TLR/RLR in HepaRG cells was similar to PHH. Most of receptors, except for TLR-7 and 9, were expressed as proteins and functionally active as evidenced by the induction of some innate genes, as well as by secretion of IL-6 and IP-10, upon agonist stimulation. The highest levels of IL-6 and IP-10 secretion were obtained by TLR-2 and TLR-3 agonisation respectively. The highest preventive anti-HBV activity was obtained following TLR-2, TLR-4 or RIG-I/MDA-5 stimulations, which correlated with their high capacity to produce both cytokines.

Conclusions: Our results indicate that HepaRG cells express a similar pattern of functional TLR/RLR as compared to PHH, thus qualifying HepaRG cells as a surrogate model to study pathogen interactions with hepatocyte innate system.

101 **Introduction**

102 The liver is targeted by several important human pathogens, including plasmodium
103 falciparum or hepatotropic viruses [1]. Among them, Hepatitis A virus (HAV) and HEV
104 do not usually establish persistent infections, whereas HBV, HDV and HCV may
105 evolve toward chronicity [1]. The high number of chronic carriers, around 240 million
106 for HBV (WHO data) and 130 million for HCV worldwide, account for the rapid
107 increase in the incidence of hepatocellular carcinoma (HCC) [2]. Pathogenesis of
108 HBV and HCV infections, and the severity of virus induced liver injury are mainly
109 determined by immunologically-mediated events rather than the virus itself, since
110 only limited cytopathic effects are noticed in hepatocytes following infection [3].

111 Infection of cells by microorganisms leads to the activation of the host
112 inflammatory defense response through the initial sensing of pathogen mediated by
113 innate pattern recognition receptors (PRR) [4]. PRR are mainly represented by Toll-
114 like receptors (TLR), C-type lectin receptors (CLR), RIG-I-like receptors (RLR) and
115 NOD-like receptors (NLR). In addition, intracellular DNA sensors are proposed as
116 new types of PRR [5]. To date, ten functional TLR have been identified in human
117 (TLR-1 to 10) and detect specific pathogen-associated molecular patterns (PAMP)
118 derived from microorganisms [6]. Upon recognition of respective PAMP, TLRs recruit
119 distinct sets of adaptor molecules which initiate downstream signaling events that
120 lead to the secretion of inflammatory cytokines, type-I IFN, chemokines and
121 antimicrobial peptides [7]. *In vitro* models have shown that an increasing number of
122 viruses can activate TLR pathways [8]. Cells also express cytoplasmic RNA
123 helicases such as RIG-I (Retinoic-acid Inducible Gene-I) and MDA-5 (melanoma-
124 differentiation-associated gene-5) that function as an alternative class of PRR
125 through the recognition of dsRNA produced during viral replication.

126 Viruses can evade IFN response and recent studies have increased our
127 understanding of the underlying molecular mechanisms of inhibitions [9]. Following
128 infection, viruses can induce complex intracellular events that affect many
129 components of host signaling pathways to its own benefits [10, 11]. Elucidating the
130 strategies that are used by viruses to inhibit host responses is important for the
131 understanding of the mechanism(s) by which viruses can establish persistent
132 infections and to design strategies to unlock this inhibition for therapeutic purposes.

133 Although the expression of some PRR genes was shown in human liver tissue and
134 PHH, the expression and functional activity of a wide spectrum of PRR on human
135 hepatocytes remains poorly defined [12, 13]. Transformed hepatic cell lines such as
136 HepG2 and Huh7 are commonly used to study hepatotropic viruses life cycle and
137 early host/virus interaction, however, in terms of innate immunity, these cells differ
138 from PHH in that they do not have fully functional IFN pathways [14, 15] and display
139 profound defect in PRR signaling [16]. Beside recently developed models based
140 hepatoma cells overexpressing NTCP [17], or on the complex use of induced
141 pluripotent cells (iPS) [18], only freshly prepared primary human hepatocytes and the
142 hepatocyte-like HepaRG cells can support a complete HBV and HDV life cycle,
143 including the early events of infection [19-21].

144 HepaRG cells are bipotent liver progenitor cells that differentiate into both
145 cholangiocyte-like and hepatocyte-like cells in culture. Throughout differentiation,
146 HepaRG cells evolve from a homogeneous dedifferentiated, depolarized epithelial
147 phenotype showing no specific organization to a structurally well-defined and
148 polarized monolayer closely resembling those formed in PHH culture [22-24]. This
149 cell line represents an *in vitro* human model for hepatocytic differentiation and was
150 shown to be functional for IFN signaling [25]. However, the functionality of
151 TLRs/RLRs this cell line has not been described so far.

152

153 ## Material and Methods

154 ### *Cell culture and HBV infection*

155 HepaRG cells were cultured and differentiated in presence of DMSO (cell culture
156 grade, Sigma) as previously described [20]. Primary human hepatocytes (PHH) were
157 freshly prepared, as previously described and maintained in same media as
158 dHepaRG [26]. Huh7 and HepG2 (ATCC) cells were also maintained in complete
159 William's medium and cultured in presence of DMSO for three days before RNA
160 extraction. The transfection of siRNA (SMARTpool™ from Darmacon/GE) into
161 dHeppaRg or PHH cells was performed using Darmafect-1 reagent, as
162 recommended by provider (Darmacon/GE). HBV recombinant virus (i.e. inoculum)
163 was produced in HepG2.2.15 cells and differentiated HepaRG infected as previously
164 described [27].

165 ### *Nucleic acid extraction and RT-qPCR/qPCR*

166 Total RNA was extracted from cells with the NucleoSpin RNA II kit according to
167 manufacturer's instructions (Macherey-Nagel). RNA reverse transcription was
168 performed using the Superscript III RT (Life Technologies). cDNA was then analyzed
169 by qPCR with the "Express SYBR GreenER™ qPCR SuperMix Universal"
170 (Invitrogen). RNA expression level was determined using the comparative cycle
171 threshold (Ct) method, where the amount of target DNA was normalized to
172 housekeeping genes *rplp0* and *β-actin* cDNA ($2^{-\Delta Ct}$). When cells were stimulated with
173 ligands the amount of target DNA was normalized to housekeeping genes and to the
174 unstimulated control cDNA ($2^{-\Delta\Delta Ct}$). Primers used for the qPCR step are presented in
175 **Table 1**. Total DNA was extracted from HepaRG using the MasterPure™ Complete
176 DNA/RNA Purification Kit (Epicentre). Quantitative PCR for intracellular HBV DNA
177 was performed as indicated above using HBV specific primers.

178 ### *Toll-and RIG-like receptor agonists and stimulation*

179 All agonists were purchased from InvivoGen and used according to provider's
180 recommendation. Pam3CSK4, LTA, Poly(I:C)-LMW, LPS, Flagellin (FLA-BS), FSL-1,
181 Imiquimod (R837) or CL264, R848, ssRNA40/lyoVec^{TM}, CpG ODN-2395 (class-C +
182 CpG control), Poly(I:C)-LMW/LyoVec^{TM} were respectively used to stimulate/engage
183 TLR-1/2, TLR-2, TLR-3, TLR-4, TLR-5, TLR-2/6, TLR-7, TLR7/8, TLR-8, TLR-9, and
184 RIG-I/MDA-5. The concentrations used are indicated in the figure legends.

185 ***Flow cytometry analysis***

186 To detect TLR expression, 10^6 HepaRG or PHH cells were fixed with
187 paraformaldehyde (2%; Sigma) and permeabilized with saponin (0.25%; Sigma).
188 After saturation (saponin 0.25%, fraction-V albumin 3%; Sigma), cells were stained
189 with purified anti-human TLR-2, 4, 5, 6 (sc-73181, sc-52062, sc-57461, sc-30001;
190 Santa Cruz), anti-human TLR-7, 8, 9 (Alexis Biochemicals (ALX-210-874-C100, ALX-
191 804-376-C100) or Abcam (ab184943, ab85859, ab45371)), or anti-human TLR-3
192 (mAb hTLR-3; Invivogen), followed by incubation with the appropriate secondary
193 antibody conjugated to Alexa Fluor 488 (Invitrogen). For double staining, either anti-
194 cytochrome P450-3A4 antibody (ab135813; Abcam) or anti-cytochrome P450-3A1
195 (ab22724; Abcam), which specifically stain hepatocytes and not cholangiocytes
196 **(Sup.Fig. 1)** were used in combination with anti-PRR antibodies from either mouse
197 or rabbit origin. Cells were analyzed using a FACScalibur flow cytometer and
198 analyzed using CellQuestPro software (BD Biosciences).

199 ***Western blot analysis***

200 Cells were harvested in lysis buffer (140 mM NaCl, 10 mM Tris pH 7.6, 1 mM
201 EDTA, 1% Triton X-100, 0.05% sodium dodecyl sulfate, 1x protease inhibitor cocktail
202 5Roche). Clarified lysates were subjected to SDS-PAGE and Western blot transfer to
203 nitrocellulose membranes (Millipore). Membranes were probed with anti-RIG-I, anti-
204 MDA5, or anti-TLR2 antibodies (Alexis Biochemicals and Santa Cruz) followed by

205 peroxidase-conjugated secondary antibodies (Life Technologies) and detection by
206 chemiluminescence (Pierce). Anti-actin (Sigma) was used as a loading control.

207 ***Enzyme-linked immunoassay for IL-6, IP-10, and secreted HBV antigens***

208 Supernatant from stimulated culture was harvested and assayed for IL-6 and IP10
209 protein by ELISA using the human IL-6 or human IP10 assay kit according to the
210 manufacturer's protocol (Invitrogen). Cut-offs for these ELISA were 2.5 pg/mL.
211 HBeAg and HBsAg were detected in the supernatant of infected HepaRG by ELISA
212 using Autobio kits (Autobio, China). Cut-offs for these ELISA were respectively 1
213 NCU/mL (i.e. 1 NCU ≈ 13 ng) and 2.5 ng/mL.

214 ***Statistical analysis***

215 Statistical analysis was performed using the nonparametric Mann-Whitney tests
216 using the GraphPad Prism software. For all tests, a p value ≤0.05 (*) was considered
217 as significant.

218

219

220 ## Results

221 ***Analysis of TLR and RLR RNA expression in dHepaRG cells and PHH at RNA***
222 ***level***

223 Differentiated HepaRG cells, PHH, as well as DMSO-partially re-differentiated
224 Huh7 [28] and HepG2 [29] cells were investigated for total RNA expression levels for
225 *tlr-2* to *tlr-10*, as well as *rig-I* and *mda-5*. All *tlrs* were similarly expressed in dHepaRG
226 and PHH, whereas levels of expression were significantly lower in the two
227 transformed hepatoma cell lines, except for *tlr-8* expression, which was slightly
228 higher in the latters. *Rig-I* and *mda-5* were well expressed in all cell types, but more
229 expressed in PHH as compared to other cells. *Tlr-7*, *8*, *9*, and *tlr-10* were less
230 expressed than other sensors in both dHepaRG and PHH **(Fig. 1A and 1B)**. The
231 expression level of these PRR was further analyzed according to the HepaRG
232 differentiation status **(Fig.1C)**. Differentiated HepaRG cells expressed slightly more
233 *tlr-2, 3, 4* and *tlr-6* as compared to proliferative HepaRG (pHepaRG) whereas the
234 expression level of the other sensors tested was not highly significantly changed in
235 dHepaRG versus pHepaRG.

236 ***Analysis of TLR and RLR protein production in HepaRG cells and PHH***

237 TLR protein expression in pHepaRG and dHepaRG was further analyzed **(Fig.**
238 **2A/2B)**. FACS analysis showed that all TLR (TLR-2 to 9, with TLR-6 being less
239 expressed) were produced in both proliferative and differentiated HepaRG cells **(Fig.**
240 **2A)**, and a positive reactivity was also obtained for TLR-7, 8, and 9, whose
241 expression at RNA level was very low. The expression of TLR in dHepaRG cells was
242 found qualitatively similar, but quantitatively slightly higher as compared to PHH (two
243 different batches) **(Fig. 2B and Sup.Fig. 2)**, whereas the RNA levels were similar
244 suggesting differences in post-transcriptional or post-translational regulations. The
245 expression of the cytosolic sensors RIG-I and MDA-5 was analyzed by western-blot

246 in PHH and differentiated HepaRG cells. Both sensors could be detected at basal
247 level, but were highly expressed following cell stimulation (24h) with IFN-α (1000
248 UI/ml) **(Fig. 2C)**. Since differentiation of HepaRG cells leads to a mixture of
249 hepatocyte- and cholangiocyte-like cells, we sought to determine whether expression
250 of PRR would be differentially distributed in each cell type by flow cytometry using a
251 double staining with antibodies directed against selected TLR and hepatocyte
252 specifically-expressed P450 cytochromes. We found that the selected TLR were
253 more expressed in hepatocyte-like cells, as compared to cholangiocyte-like ones
254 **(Fig. 2D)**.

255 ***Inducibility of cytokine RNA expression upon stimulation with TLR/RLR***
256 ***prototypic ligands/agonists***

257 Differentiated HepaRG cells were stimulated for 2, 4, 8 and 16h by indicated
258 TLR/RLR cognate ligands at concentrations indicated in legend. This stimulation
259 could induce the expression of the pro-inflammatory cytokine gene (IL-6), the type I
260 and type III IFNs (IFNα, β and λ) and interferon stimulated genes, ISGs (ISG56). With
261 the exception of TLR7 and 9 ligands, all cognate ligands could induce IL-6 gene
262 expression at the concentration tested **(Fig. 3A, panel IL-6)**. A strong IL-6 induction
263 was observed after 16h of stimulation for TLR-2, 3, 4, 5, 6, RIG-I/MDA-5, whereas an
264 induction was observed for TLR-8 after 2h of stimulation only which returned to
265 baseline very rapidly. Because production of type-I interferons, in particular IFN-β, is
266 one of the first cellular antiviral defenses, we tested whether this gene was activated
267 at RNA level in HepaRG cells after some TLR/RLR stimulations. Indeed interferon-β
268 gene expression was found induced after stimulation by some cognate ligands **(Fig.**
269 **3A, panel IFN-β)**. IFN-β gene moderately increased after stimulation with TLR-3, 7, 8
270 and 9 ligands, but strongly increased after stimulation with RIG-I/MDA-5 ligands. It is
271 worth noting that the concentration of poly(I:C)-LMW/lyoVec^{TM} (1μg/mL) used in
272 these experiments was shown to be slightly toxic when multiple dosing were used for

273 antiviral studies; this led to a readjustment of the concentration in experiments
274 presented hereafter. Stimulation of TLR-3, 7, 9 and RIG-I/MDA-5 also slightly
275 induced IFN-α expression **(Fig. 3A, panel IFN-α)**. Type-III IFN expression has been
276 shown to depend on the same stimulations, i.e. viral infection or ds-RNA ligands, and
277 signal transduction pathway, as those inducing type I IFN expression in epithelial
278 cells [30]. IL-28B (IFN-λ3) expression was only induced by TLR-3 or RIG-I/MDA-5
279 stimulations, although the kinetic of activation was different (i.e. earlier for TLR-3)
280 **(Fig. 3A, panel IL-28B)**. The induction of IL-29 (IFN-λ1) was unexpectedly lower
281 than that of IL-28B **(Fig. 3A, panel IL-29).** The activation of all IFN transcripts was
282 associated to the activation of prototypic ISGs such as ISG56 induced after TLR-3
283 stimulation and RIG-I/MDA-5.

284 In PHH, low-level of gene activation was also observed with TLR-7/9-L **(Fig. 4A)**,
285 but as PHH cultures are not completely pure, we cannot exclude that residual
286 activation was due to the presence of other liver cells.

287 ***Secretion profile of IL-6, IP-10, and type-I IFN after stimulation with TLR/RLR***
288 ***prototypic ligands***

289 The cell culture supernatants of HepaRG cells were first tested for the production
290 of IL-6 and IP-10 following a 24h TLR/RLR stimulation with increasing doses of
291 prototypic ligands **(Fig. 3B/C)**. Basically, all TLR/RLR ligands tested, but not TLR-
292 7/8/9 ligands, could induce a strong secretion of IL-6 in supernatants in a dose
293 dependent manner. The highest IL-6 secretions (between 500 and 1000 pg/mL) were
294 obtained with of TLR-1/2, 4, 6 ligands used at a high concentration, as expected.
295 Double-stranded RNA ligands, engaging either TLR-3 or RIG-I/MDA-5, led to a lower,
296 but yet significant level of IL-6 production (i.e. between 300 and 400 pg/mL). Despite
297 a good RNA expression induction, TLR-8 ligands did not lead to a strong
298 accumulation of IL-6 in the supernatant (only around 30 pg/mL). This could be due to

299 fast turnover of the secreted cytokine after a very early expression of IL-6 at RNA
300 level. In agreement with RNA expression data, TLR-7 and 9 ligands did not lead to
301 any detectable level of IL-6. The same ligands that were able to induce IL-6
302 secretion could also increase the IP-10 production . However, TLR-3 and RIG-I/MDA-
303 5 ligands led to the higher production, with a respective secretion of around 2800 and
304 1400 pg/mL at the highest doses of ligands, as compared to the secretion of around
305 800 pg/mL with TLR-1/2 or 4, and respectively 200 and 500 pg/mL with TLR-5 and 6
306 ligands. Similarly to what was obtained with IL-6 secretion, TLR-7, 8 and 9 did not
307 lead to any detectable level of IP-10. Results obtained in PHH are shown in **Figure**
308 **4B**. In PHH even more IP-10, but less IL-6 (with the exception of TLR-4 agonisation)
309 were produced as compared to dHepaRG.

310 In contrast, we could only detect biologically active type-I IFN, using a functional
311 assay, in the supernatant of HepaRG cell stimulated only with very high (and rather
312 cytotoxic in the context of multiple dosing; **data not shown**) doses of RIG-I and
313 MDA/5 ligands **(Sup.Fig. 3)**, thus suggesting that the interferon levels produced after
314 stimulation by other prototypic ligands might be under the detection threshold of the
315 assay.

316 ***Exemplary comparison of expression and functionality of TLR-2 in dHepaRG***
317 ***and PHH***

318 As a first attempt to compare the expression and functionality of all TLRs/RLRs in
319 dHepaRg and PHH, we studied TLR-2 expression and function in both cell type using
320 two additional batches of cells. Using Pam3CSK4 to stimulate and specific TLR-2
321 siRNA (siRNA against HCV was used as control) to control specificity, we have
322 shown that i) the basal level of expression (RNA and protein) of this sensor is rather
323 even in both cell types **(Fig. 5A/5B; Sup.Fig. 2)**, ii) its expression can be strongly
324 induced by Pam3CSK4 agonisation and decreased by specific siRNA **(Fig.**

325 **5A/5B/5C)**, and iii) its specific agonisation by ligands leads to a strong secretion of
326 IL-10 (slightly stronger in PHH as compared to dHepaRG) **(Fig. 5D)**. Thus this
327 pathway seems to be operational in hepatocytes.

328 ***Antiviral activity of some TLR/RLR ligands in HBV-infected dHepaRG***

329 To fully characterize the functionality of the TLR/RLR receptors that are expressed in
330 HepaRG cells, we have treated cells with TLR-1/2, 3, 4, 7, 8, 9 or RIG-I/MDA-5
331 ligands with non-toxic doses (as determined by 3x exposure of HepaRG cells to
332 ligands; **Fig. 6D**) for 24 hour before infecting them with HBV. A 24 hours inoculation
333 with HBV was made in presence of cytokine produced during the 24h exposure to
334 ligands. Then, infected cells were washed and further retreated twice with the same
335 dose of ligands respectively at days 1 and 4 post-infection. The impact of TLR/RLR
336 ligands treatment on the establishment of HBV infection was analyzed at day 7 post-
337 infection by measuring HBV viral protein secretion (HBsAg/HBeAg) and the
338 accumulation of intracellular HBV DNA by qPCR. A strong antiviral activity was
339 obtained with TLR-1/2 and TLR-4 ligands, as well as with a RIG-I/MDA-5 ligand **(Fig.**
340 **6A/B/C)**, whereas weaker or no antiviral activity was observed with a TLR-3, 7, and 8
341 ligands. Surprisingly, an antiviral activity was obtained with the TLR-9 ligand/agonist,
342 which did not correlate with any production of IL-6 or IP-10. However the same
343 antiviral activity was obtained with the control CpG ligand, thus suggesting that the
344 phenotype obtained was independent of innate functions.

345

346 ## Discussion

347 Hepatoma cell lines or transformed human hepatocytes are commonly used to
348 study hepatotropic pathogens, however the relevance of these models is limited
349 when studying the interplay between pathogens and hepatocyte innate immunity.
350 Indeed pathogen sensing, IFN response and NF-κB signaling are commonly altered
351 these cell types as compared to PHH [16]. RNA levels for TLR receptors and a set of
352 downstream signaling molecules are lower in these cells compared to PHH, and not
353 modified in response to stimulation. Moreover, we previously showed that a type-I
354 IFN response to HBV replication does not lead to an antiviral effect in transformed
355 HepG2 cells while it suppressed viral replication in differentiated HepaRG cells [31].
356 Furthermore it has been observed that the patterns of cytokine secretion were also
357 different even when a common transcription factor (NF-κB) was involved. The defects
358 in TLR signaling observed in hepatoma cells was consistent with the hypothesis that
359 innate immunity could play an important role in suppressing tumorigenesis [16].
360 These defects make them of limited interest to study innate immune response
361 induced by an hepatotropic pathogen [14, 15].

362 In contrast, PHH and HepaRG cells, which are mainly non-cancerous, functional
363 for IFN signaling, and permissive to HBV replication, are more relevant models [20,
364 21]. Their transcriptomes have been shown to be closer to each other, as compared
365 to hepatoma cells, and closer to that of liver biopsies [32]. The relatively low
366 replication rate of hepatotropic viruses (low rate for HBV and virtually no permissivity
367 to HCV) in HepaRG cells might result, at least in part, from a cellular antiviral
368 response [33]. Indeed, an experimentally strong HBV expression and replication after
369 baculoviral delivery of HBV was shown to induce a potent anti-HBV IFN response,
370 while in the same experimental conditions this IFN response did not show any
371 antiviral effect in transformed HepG2 cells [31]. Therefore HepaRG cells, because

372 physiologically related to PHH [34], were expected to be a valuable tool to study
373 host/pathogen interactions. However, detailed features of this immune response
374 were still unclear and the present study provides the first characterization and
375 functionality of PRRs gene expression in an HBV susceptible and non-cancerous cell
376 line, the HepaRG cell in comparison with PHH.

377 In this respect, our study showed that RNA of all TLR as well as cytoplasmic
378 helicases RIG-I and MDA5 receptors, could be detected in either proliferative or
379 differentiated HepaRG cells, in similar amount than in PHH. While all RNA could be
380 detected, the level of expression of *tlr-7, 8, 9* and *tlr-10* proved to be very low in
381 hepatocytes. Protein expression analysis showed that all TLR were expressed in
382 both dHepaRG and PHH, with slightly higher levels of expression in HepaRG. The
383 detection of TLR-7, 8, and 9 at protein level contrasted with the low RNA expression,
384 and suggests that post-transcriptional mechanisms could be involved to allow
385 detectable protein expression. RIGI and MDA5 were also expressed at the protein
386 level and highly inducible after interferon stimulation. For most PRR, the stimulation
387 by their prototypic ligands could induce their own expression by positive feed-back.
388 These stimulations could also lead to the RNA induction of several cytokines such as
389 IL-6, with the exception of TLR-7 and 9. However, TLR-7 and 9 ligands could
390 somehow induce a weak IFN-α and β RNA expression implying that all these TLR
391 could be slightly functional, yet not leading to detectable secretion of IL-6 or IP-10. In
392 humans, the expression of TLR-7 and TLR9- is mainly confined to plasmacytoid
393 dendritic cells and B-lymphocytes. However, low levels of TLR7 and or TLR9 have
394 been reported in other cell types, including hepatocytes [35] particularly in the setting
395 of chronic inflammation [36]. In addition, the functionality of TLR-3 and RIG-I/MDA-5
396 pathway was demonstrated after cell stimulation with extracellular dsRNA (poly(I:C))
397 and cytoplasmic dsRNA (poly(I:C)-lyovec) respectively showing an induction of
398 IFNα, β and λ as well as ISGs (such as ISG56). More importantly, after stimulation

399 with prototypic ligands targeting PRR, using different doses of ligands, IL-6 could be
400 secreted and detected at high levels up to 1000 pg/ml with TLR-1/2, 4, and 6
401 stimulations, and between 100 and 500 pg/mL for TLR-3, 5, as well as RLR
402 stimulations. A weak IL-6 expression was obtained with TLR-8 stimulation, but no
403 secretion could be evidenced for TLR-9 and TLR-7 ligands. In contrast, dsRNA
404 ligands engaging either TLR-3 or RIG-I/MDA-5 were more prone to induce secretion
405 of IP-10 (i.e. with production of 2800 and 1400 pg/mL respectively), an IFN-related
406 chemokine. Other functional PRR, based on their ability to induce IL-6 secretion,
407 were also capable to induce IP-10. Interestingly, type-I interferon could only be
408 detected in cell supernatants after stimulation with high, yet rather toxic doses of
409 RIG-I and MDA-5 ligands, thus suggesting that it is difficult to measure the production
410 of type-I IFN by hepatocytes.

411 An important part of the demonstration of PRR functionality in HepaRG relies on
412 the antiviral effect observed with some ligands regarding the establishment of HBV
413 infection in these HepaRG cells. Indeed, while it was shown that IL-6 could have an
414 antiviral effect against HBV [37], many other cytokine may also have antiviral effect.
415 Therefore, the engagement of PRR in HepaRG was expected to lead to conditioned
416 media having potent antiviral activity. This was strongly obtained with TLR-1/2 and 4
417 agonists, as well as with RIG-I/MDA-5 ligands, but surprisingly less with a TLR-3
418 ligand, despite its ability to better induce the production of IL-6 and IP-10 as
419 compared to a RIG-I/MDA5 agonist. This result could only be explained by the ability
420 of RIG-I/MDA-5 agonists to induce measurable amount of type-I IFNs compared to
421 TLR-3 agonists. The strongest antiviral activity was obtained by Pam3CSK4
422 agonisation of TLR-1/2, a pathway which is well functional in hepatocytes, as shown
423 by more advanced comparison done in both dHepaRG and PHH **(Fig. 5)**. These
424 interesting results warrant further investigation on this pathway and its antiviral
425 effectors.

426 Infections by hepatotropic pathogens can be cleared once innate immune
427 activation and pathogen-specific T cell responses predominate over pathogen
428 immune escape mechanisms, as it occurs in HAV infections, and in cases of
429 resolution of acute HBV or HCV infections. Viral persistence in hepatocytes can
430 occur by escaping both innate and adaptive immune responses, as observed in
431 chronic HBV or HCV infections. HepaRG exhibits functional TLR and dsRNA-
432 activated signaling pathways in contrast to cultured hepatoma cells [38]. It appears
433 unclear why hepatoma cell lines are unresponsive to a wide range of TLR agonists,
434 but RNA levels for TLR receptor and a set of downstream signaling molecules
435 appear to be lower in hepatoma cell lines than in primary hepatocytes [16]. However,
436 low responsiveness to ligands was observed even when the cognate TLR was
437 expressed at apparently normal levels, suggesting that other downstream factors
438 must be involved. Our data show that HepaRG cells express functional PRRs. The
439 activation of these PRRs leads to the production of cytokines/chemokines, including
440 cytokines bearing antiviral activity. This study opens doors to relevant and detailed
441 analyses of hepatocyte-like cell response to hepatotropic viruses or other
442 hepatotropic pathogens in the absence of non-parenchymal or liver resident immune
443 cells. Therefore, the HepaRG cell line that maintains the functional properties of
444 primary human hepatocytes provides a unique tool for the understanding of the
445 interplay between the innate immune response and hepatotropic pathogens. This
446 work may have potential applications to study the activation of hepatocyte innate
447 immunity by these pathogens, the evasion to innate immunity by these pathogens,
448 and mechanism by which innate responses could be restored.
449

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539 factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. Journal of virology
540 2003;77:1092-1104.

541

542 Table 1. Primer sequences used for RT-qPCR

543

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
TLR1	caggccctcttcctcgttaga	ttcctaaaggtagaagctgttcttca
TLR2	ctctcggtgtcggaat	cccgctcactgtaagaa
TLR3	tttgcgaagaggaatgtttaaatct	cacctatccgttctttctgaactg
TLR4	tcttggtggaagttgaacg	gccacaccgggaataa
TLR5	gtaccctgactcgttct	ttctgcacccatgtga
TLR6	agtggacccagactcg	agttcgtaatggcacc
TLR8	tgagctgcgctaccac	cttctgccttcgggtt
TLR9	ggcccccggcttctt	caggagtggtccactgtcttga
TLR10	gcattcccaccaggtatcataaac	aaagcccacatttacgcctatc
RIGI	gctgatgaaggcattgacattg	cagcattactagtcagaaggaagca
MDA5	cccatgacacagaatgaacaaaa	cgagaccataacggataacaatgt
IFNα	gtgaggaaatacttccaaagaatcac	tctcatgatttctgctctgacaa
IFNβ	gccgcattgaccatgtatgaga	gagatcttcagtttcggaggtaac
IL28b	taagagggccaaagatgcctt	ctggtccaagacatccccc
IL29	gtgactttggtgctaggcttg	gcctcaggtcccaattccc
IL6	acccctgacccaaccacaaat	agctgcgcagaatgagatgagtt
ISG56	agccaacatgtcctcacagac	cttctaccactgtttcatgc
β-actin	tggcattgccgacaggatgc	tctgctggaggtggacagcga
RPLP0	caccattgaaatcctgagtgatgt	tgaccagcccaaaggagaag

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555 ## Figure legends

556 **Fig. 1. m*RNA expression of TLR and RLR in differentiated and proliferative*
557 *HepaRG.*** A and B) RT-qPCR analysis of TLR/RLR expression in dHepaRG, PHH,
558 HepG2, and Huh7. The relative RNA expression was normalized to housekeeping
559 genes *rplp0* (panel A) or *β-actin* cDNA ($2^{-\Delta Ct}$) (panel B). C) Comparative RNA
560 expression of TLR/RLR in pHepaRG and dHepaRG cells. RT-qPCR analysis of
561 TLR/RLR expression was normalized to housekeeping genes (*rplp0* and *b-actin*) and
562 relatively compared to pHepaRG cells. Results are given as a mean ± SEM of 3 (or 4
563 for PHH; n≥3) independent experiments (with biological triplicate) with 3 batches of
564 PHH from different donors.

565 **Fig. 2. *Protein expression of PRR in pHepaRG and dHepaRG cells.*** A and B) TLR
566 expression analyzed by FACS. Black histograms represent staining with the
567 indicated TLR antibody, shaded gray histograms represent the isotype control, in
568 pHepaRG, dHepaRG and PHH. C) Western-Blot analysis of RIGI and MDA5 in
569 dHepaRG (and PHH), with or without IFNα (1000 UI/mL) induction. D) Expression of
570 some TLRs in hepatocyte-like cells as compared to cholangiocyte-like cells
571 measured by FACS, and reported on graph as fold increase of MFI (using
572 cholangiocyte as reference, set at 1).

573 **Fig. 3. *Induction of PRR, cytokine, and ISG gene expression in dHepaRG cells*
574 *& secretion of IL-6 and IP-10 after stimulation with prototypic ligands.*** A) Gene
575 expression was analyzed (n=3) following 2h, 4h, 8h or 16h stimulation with prototypic
576 ligands of TLR1/2 (Pam3CSK4, 500 ng/mL), TLR3 (Poly(I:C)-LMW, 10 µg/mL) TLR4
577 (LPS, 1 µg/mL), TLR5 (FLA-BS, 10 µg/mL) TLR6 (FSL1, 5 µg/mL), TLR7 (Imiquimod,
578 10 µg/mL) TLR8 (ssRNA40, 10 µg/mL), TLR9 (CpG-2395, 5 µM) RIGI/MDA5
579 (Poly(I:C)-LMW-lyovec, 1 µg/mL). After stimulation RT-qPCR were performed on
580 targeted *prr* (self activation by cognate ligands; PRR are indicated on the x-axis), *il-6,*

581 *ifn-β, ifn-α, il-28B, il-29* and *isg56* genes. The RNA level expression was normalized
582 to housekeeping genes and relatively compared to the non-stimulated control. B and
583 C) The secretion of IL-6 and IP-10, after 24 hours of stimulation with different doses
584 of indicated ligands, was quantified by ELISA. The concentration used for the ligands
585 were: TLR-1/2 (Pam3CSK4, 500-50-5 ng/mL), TLR-3 (Poly(I:C)-LMW, 10-1-0.1
586 µg/mL), TLR-4 (LPS, 1-0.1-0.01 µg/mL), TLR-5 (Flagellin, 10-1-0.1 µg/mL), TLR-6
587 (FSL1, 5-1-0.2 µg/mL), TLR-7 (Imiquimod, 10-1-0.1 µg/mL), TLR-8 (ssRNA40, 10-2-
588 0.4 µg/mL), TLR9 (CpG, 5-1-0.2 µM) RIGI/MDA5 (Poly(I:C)-LMH-Lyovec, 1-0.2-0.04
589 µg/mL). Cut-offs for IL-6 and IP-10 ELISA assays are 2.5 pg/mL; there are
590 materialized on panels B and C by dotted lines.

591 **Fig. 4. *Induction of IL-6 and IFNs gene expression in PHH cells & secretion of***
592 ***IL-6 and IP-10 after stimulation with prototypic ligands.*** A) Gene expression was
593 analyzed (n=3) after 16h of stimulation with prototypic ligands of TLR1/2
594 (Pam3CSK4, 500 ng/mL), TLR3 (Poly(I:C)-LMW, 10 µg/mL) TLR4 (LPS, 1 µg/mL),
595 TLR5 (FLA-BS, 10 µg/mL) TLR6 (FSL1, 5 µg/mL), TLR7 (Imiquimod, 10 µg/mL)
596 TLR8 (ssRNA40, 10 µg/mL), TLR9 (CpG-2395, 5 µM) RIGI/MDA5 (Poly(I:C)-LMW-
597 lyovec, 1 µg/mL). After stimulation RT-qPCR were performed on *il-6, ifn-β, ifn-α, il-*
598 *28B,* and *il-29* genes. The RNA level expression was normalized to housekeeping
599 genes and relatively compared to the non-stimulated control. B) Secretion of IL-6 and
600 IP-10, after 24 hours of stimulation with a single dose of indicated ligands, was
601 quantified by ELISA. The concentration used for the ligands were: TLR-1/2
602 (Pam3CSK4, 500 ng/mL), TLR-3 (Poly(I:C)-LMW, 10 µg/mL), TLR-4 (LPS, 1 µg/mL),
603 TLR-5 (Flagellin, 10 µg/mL), TLR-6 (FSL1, 5 µg/mL), TLR-7 (Imiquimod, 10 µg/mL),
604 TLR-8 (ssRNA40, 10 µg/mL), TLR9 (CpG, 5 µM) RIGI/MDA5 (Poly(I:C)-LMH-Lyovec,
605 1 µg/mL). Cut-offs for IL-6 and IP-10 ELISA assays are 2.5 pg/mL.

606 **Fig. 5.** ***Comparison of the functionality of TLR-2 in dHepaRG and PHH cells.*** A)
607 dHepaRG and PHH were either not or transfected with either siRNA against TLR2 or
608 control siRNA (i.e. targeting HCV) for 24h, then treated or not for 24h with
609 Pam3CSK4 (500 ng/mL). Then RNAs were extracted and subjected to RTqPCR with
610 TLR-2 specific primers. A plasmid containing TLR-2 gene was use to calculate
611 copies/μL. B and C) Protein were extracted and subjected to WB analysis with an
612 anti-TLR2 and an anti-actin to control loading. D) ELISA was performed on
613 supernatant for the detection of IP-10. Results for (A and D) are given as a mean ±
614 SEM of 2 independent experiments (n=2, but with biological quadruplicate for each
615 experiment) and differences were considered as statistically significant to the control
616 condition when p value was ≤0.05 (*).

617 **Fig. 6.** ***Effect of stimulation of some PRR receptors by ligands on HBV infection***
618 ***establishment.*** Secreted HBsAg (A), HBeAg (B), and intracellular HBV DNA
619 accumulation (C) were measured at day 7 post-infection by ELISA and qPCR in
620 HBV-infected dHepaRG cells, which were pre-treated (24h prior HBV inoculation),
621 then twice treated post-infection (day 1 and 4 post-infection) with indicated ligands.
622 Toxicity (D) was evaluated in the same conditions by the neutral red assay. The
623 concentration used were: 500 ng/mL of Pam3C4 (TLR-1/2), 10 μg/mL of poly(I:C)-
624 LMH (TLR-3), 500 ng/mL of LPS (TLR-4), 5 μg/mL of CL264 (TLR-7), 10 μg/mL of
625 R848 (TLR-7/8), 5 μM of CpG-2395 (TLR-9), 5 μM of CpG-2395 control, 0.01 μg/mL
626 of poly(I:C)-LMW-lyovec (RIG-I/MDA-5). Results are given as a mean ± SEM of 3
627 independent experiments (each with biological triplicate) and differences were
628 considered as statistically significant to the control condition when p value was ≤0.05
629 (*). Cut-offs for HBeAg and HBsAg ELISA assays are respectively 1 NCU/mL and 2.5
630 ng/mL; there are materialized on panels A and B by dotted lines.

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Figure
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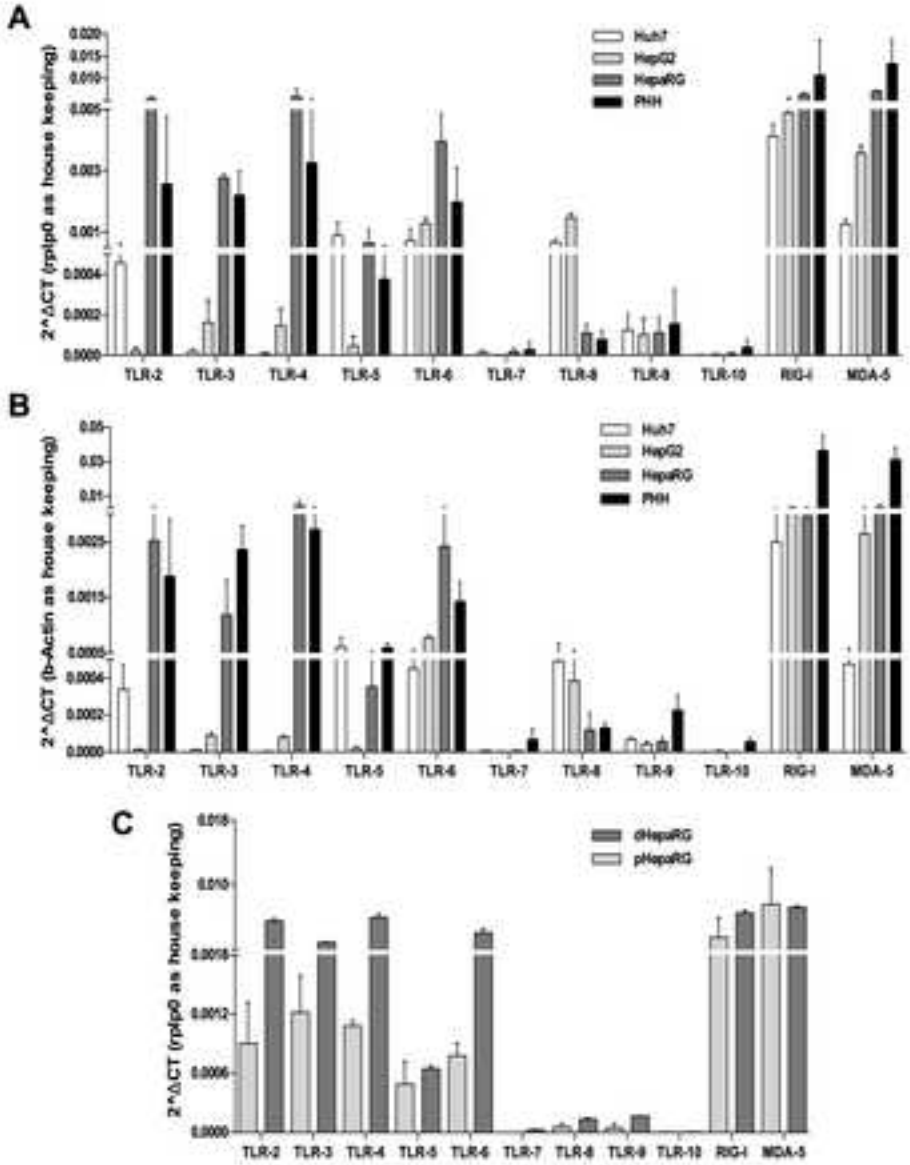
Figure 1



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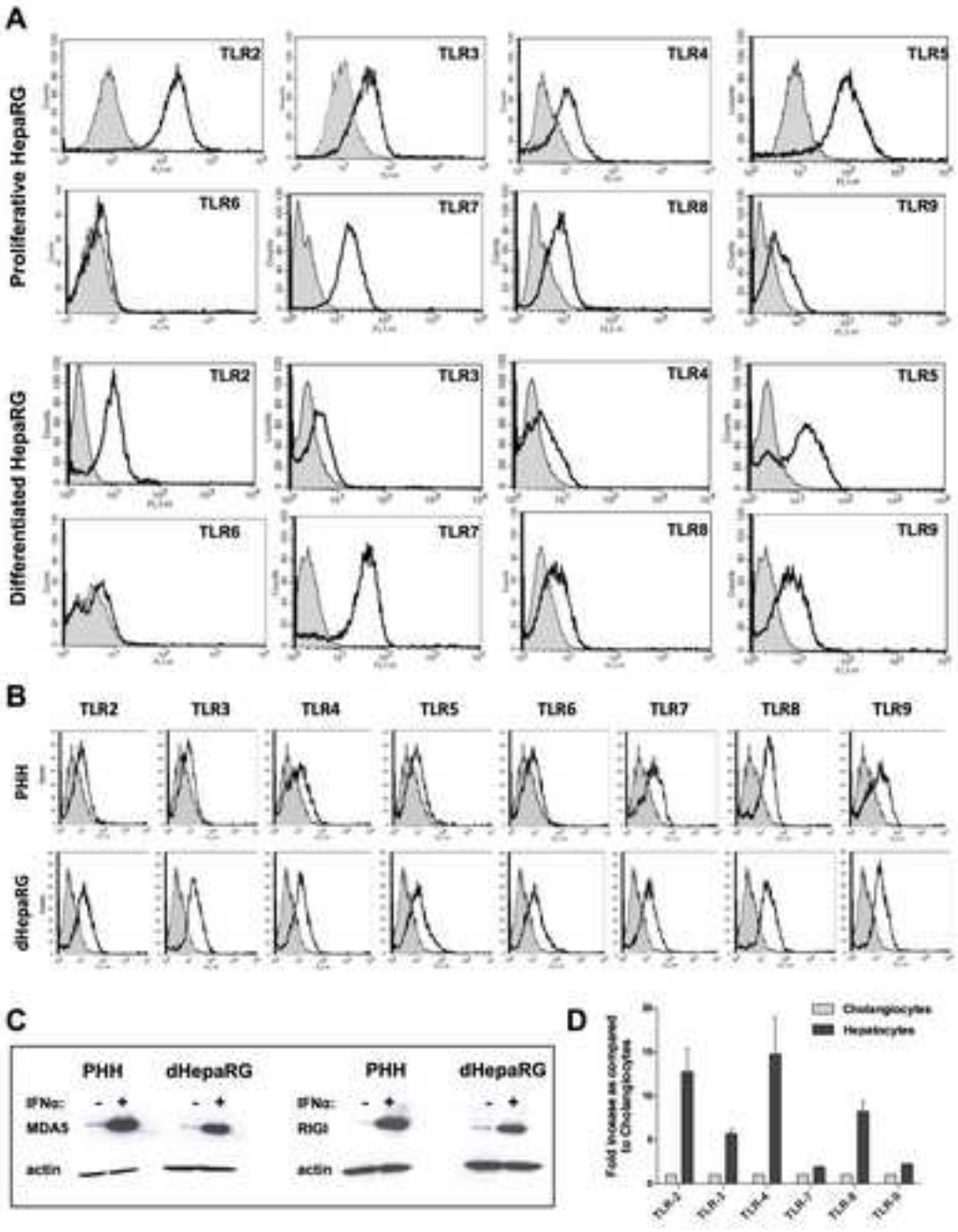

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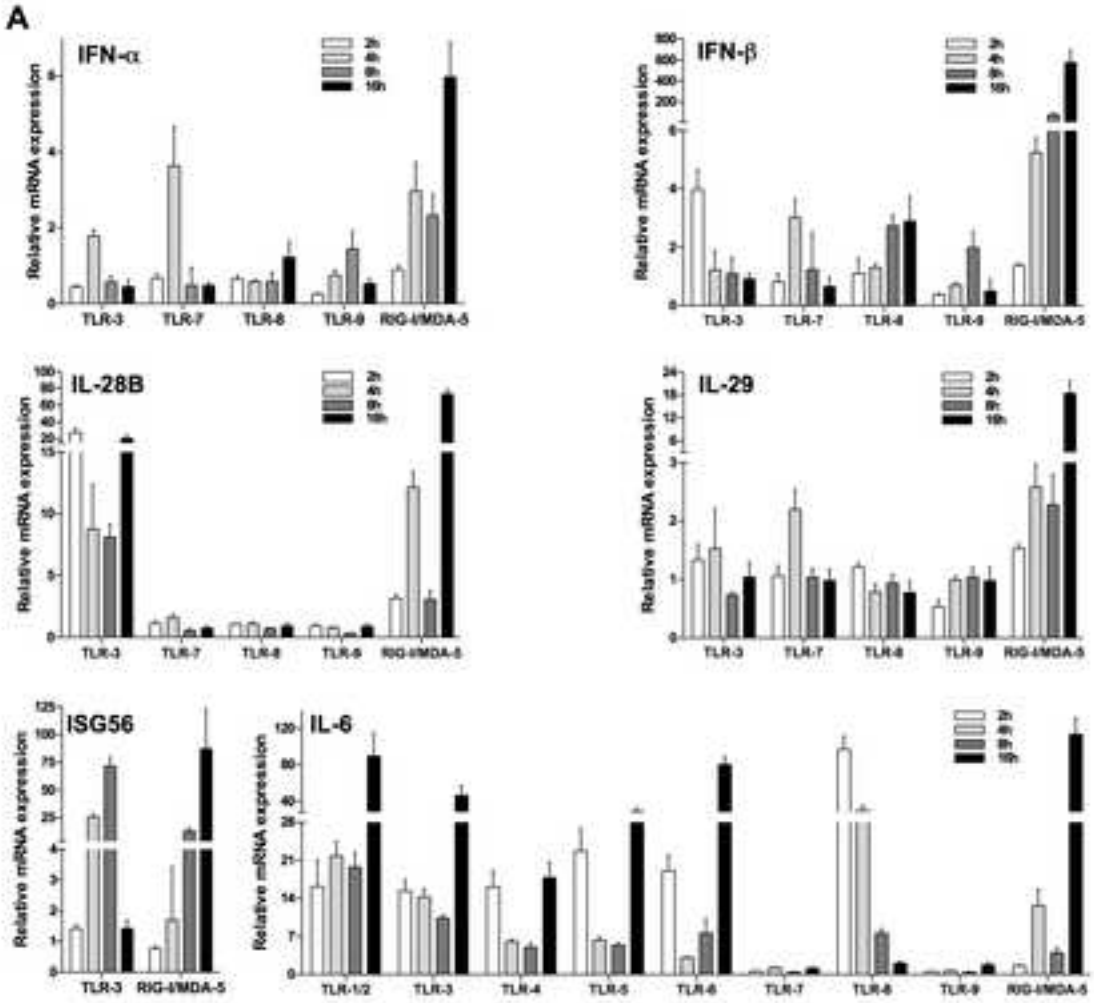

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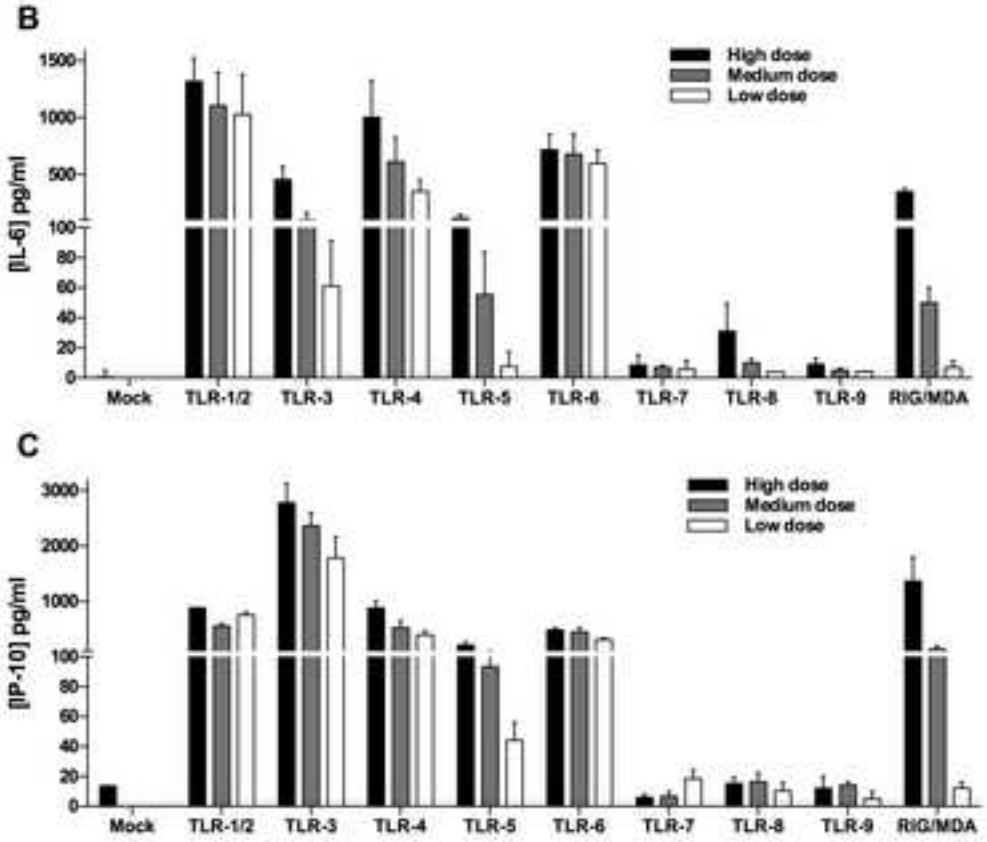
Figure 3



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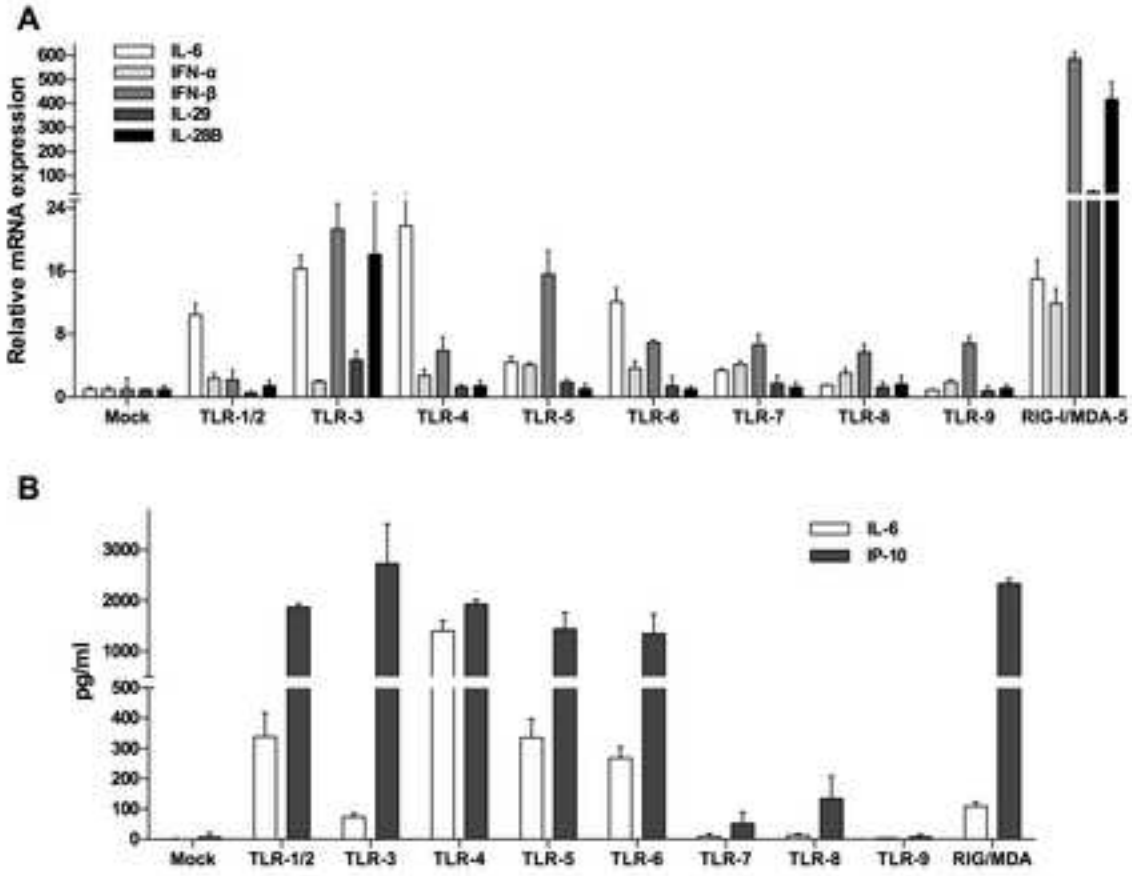

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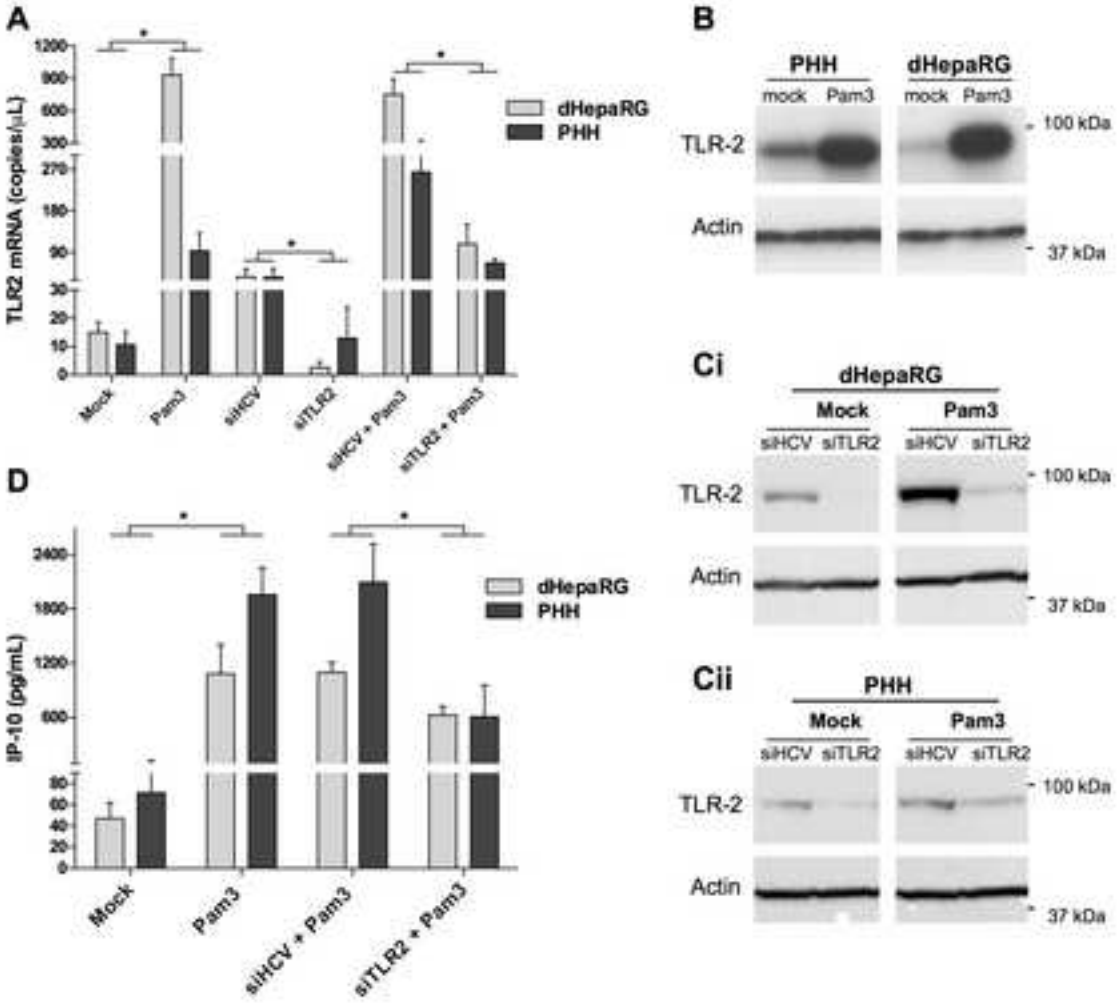

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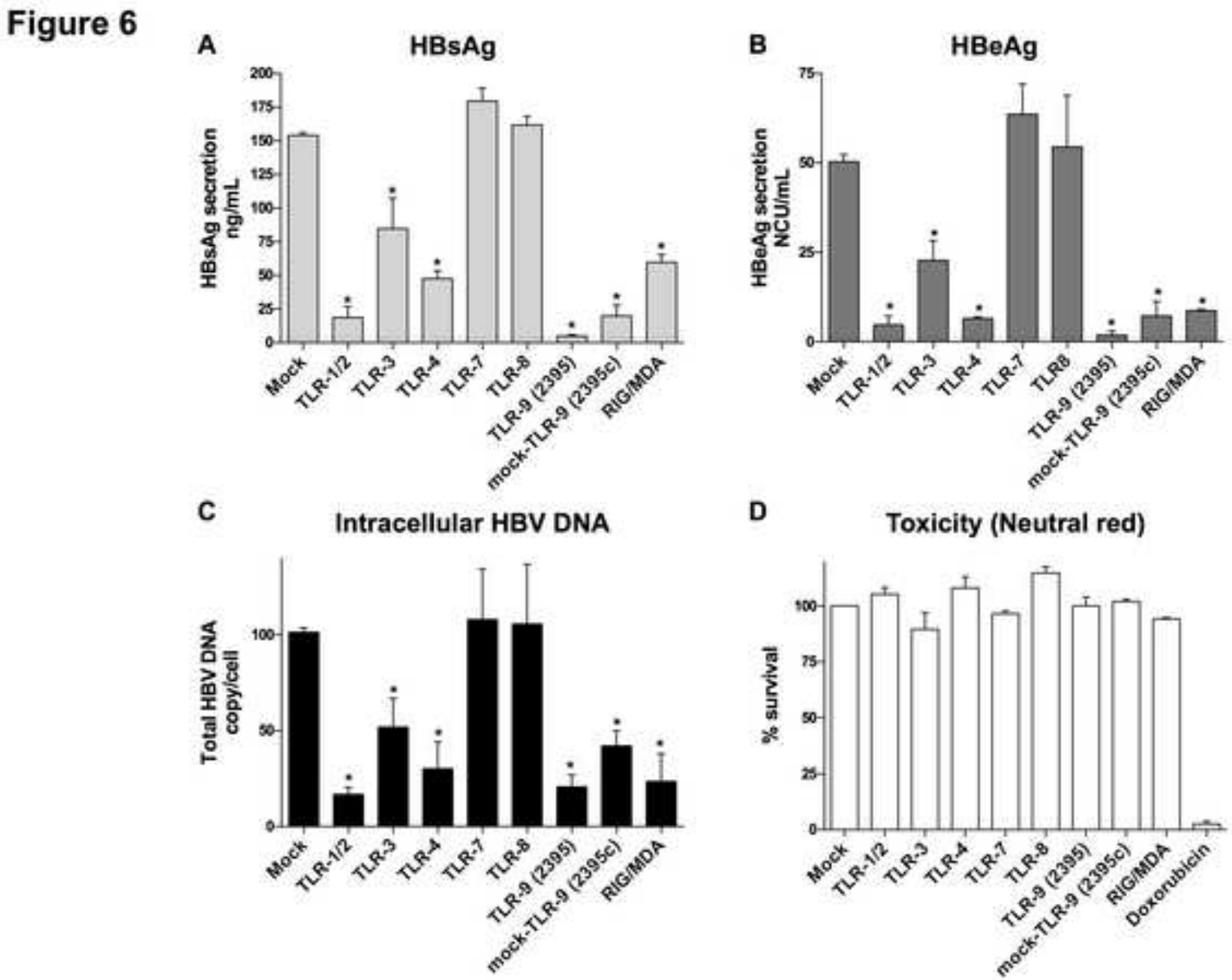


Supplementary material for the manuscript entitled "Expression and Functionality of Toll- and RIG-like receptors in HepaRG Cells" by Luangsay et al. JHEPAT-D-14-01064

Supplementary material and methods

Analysis of Secreted Type I Interferon

Two millions of Huh7.5 cells were transfected with 10 µg pISRE-Luc vector (Stratagene) in a 10 cm diameter dish using Mirus TransIT-LT1 transfection reagent according to manufacturer's instructions (Mirus Bio LLC). The pISRE-Luc plasmid (plasmid containing the luciferase gene under the control of the Interferon Stimulated Response Element) expresses luciferase under type-I IFN inducible promoter. After a 16h incubation with transfection mixture, cells were trypsinized and reseeded in a 96-well plate at approximately 3.10^4 cells/well in a volume of 50 µL. Six hours later, 50 µL of conditioned HepaRG supernatants, previously stimulated by various ligands at various concentrations as indicated. After 24h at 37°C, cells were washed with phosphate-buffered saline and lysed before luciferase activity was monitored using the Renilla Luciferase Assay System (Promega).

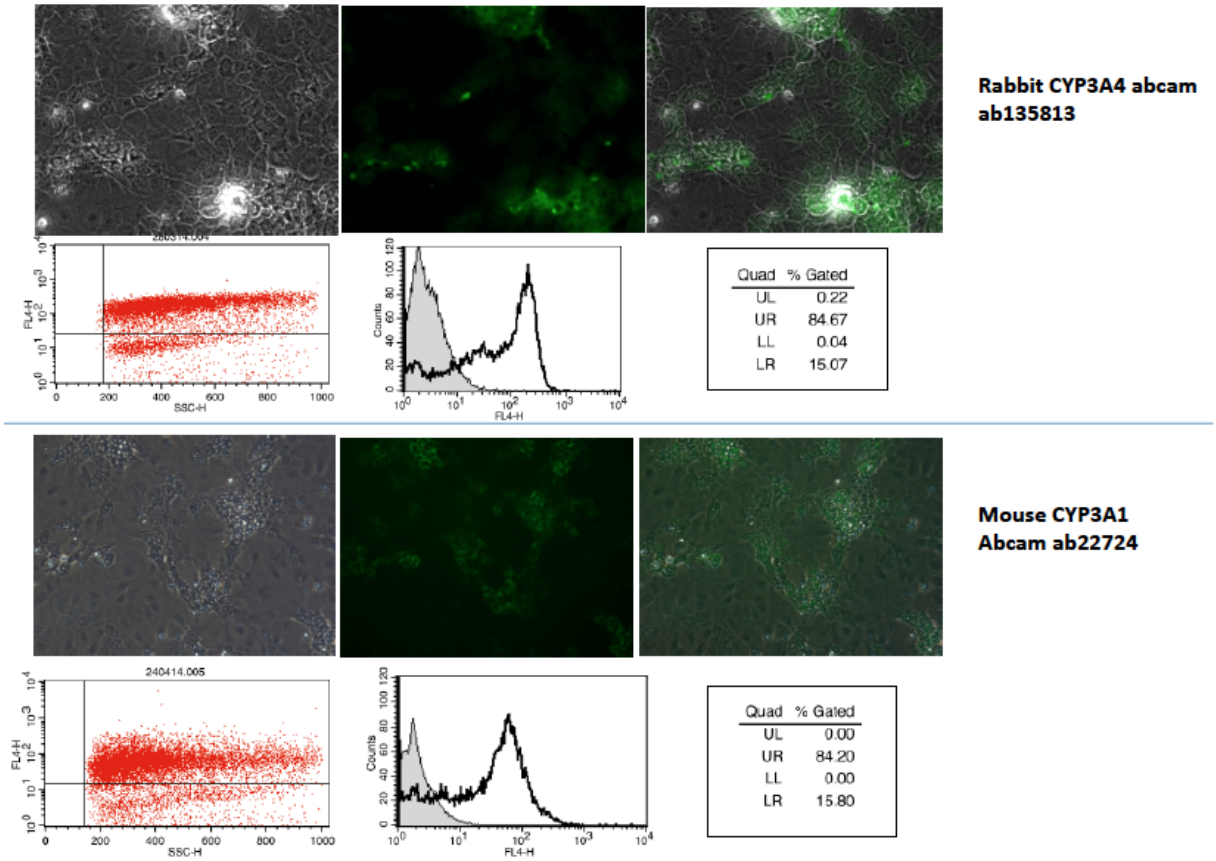
Supplementary figures and figure legends

Supplementary-Fig. 1. Cyp stainings by FACS and immunofluorescence in differentiated HepaRG. The expression of CYP3A4 (upper panel) and CYP3A1 (lower panel) was assessed by immunofluorescence and FACS in dHepaRG to evaluate the hepato-specificity and the percentage of hepatocyte-lie cells in a monolayer of dHepaRG cells.

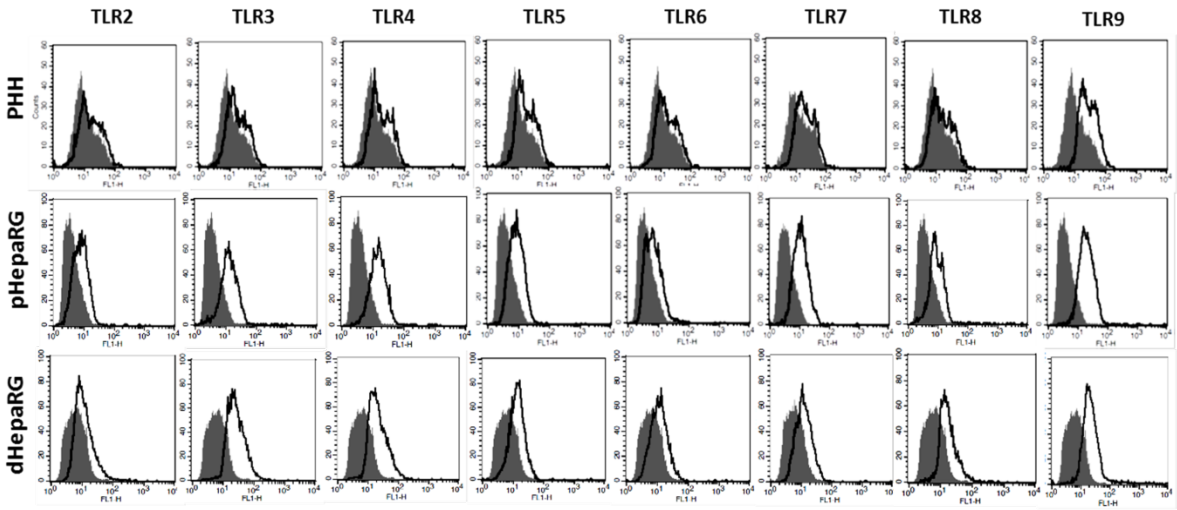
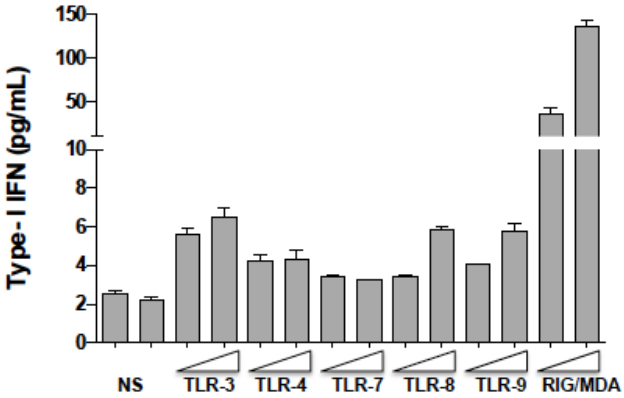
Supplementary-Fig. 2. Protein expression of PRR in pHepaRG, dHepaRG and PHH cells. TLR expression analyzed by FACS. Black histograms represent stainings with the indicated TLR antibody, shaded gray histograms represent the isotype control, in pHepaRG, dHepaRG and PHH.

Supplementary-Fig. 3. Analysis of secreted type-I IFN upon prototypic ligand treatment. Differentiated HepaRG cells were stimulated with increasing doses of PRR ligands (TLR-3 (Poly(I:C)-LMW, 1 and 10 µg/mL), TLR-4 (LPS, 0.1 and 1 µg/mL), TLR-7 (Imiquinod, 10-1-0.1 µg/mL), TLR-8 (ssRNA40, 10-2-0.4 µg/mL), TLR9 (CpG, 5-1-0.2 µM), and RIGI/MDA5 (Poly(I:C)-LMH-Lyovec, 0.2 and 1 µg/mL)). Twenty four hours after stimulation, supernatant were tested along with non stimulated cells (NS) for type I interferon activity following procedure described in supplementary materials and methods.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3