Promotion of Hepatocellular Carcinoma by the Intestinal Microbiota and TLR4

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SUMMARY

Increased translocation of intestinal bacteria is a hallmark of chronic liver disease and contributes to hepatic inflammation and fibrosis. Here we tested the hypothesis that the intestinal microbiota and Toll-like receptors (TLRs) promote hepatocellular carcinoma (HCC), a long-term consequence of chronic liver injury, inflammation, and fibrosis. Hepatocarcinogenesis in chronically injured livers depended on the intestinal microbiota and TLR4 activation in non-bone-marrow-derived resident liver cells. TLR4 and the intestinal microbiota were not required for HCC initiation but for HCC promotion, mediating increased proliferation, expression of the hepatomitogen epiregulin, and prevention of apoptosis. Gut sterilization restricted to late stages of hepatocarcinogenesis reduced HCC, suggesting that the intestinal microbiota and TLR4 represent therapeutic targets for HCC prevention in advanced liver disease.

INTRODUCTION

Increased translocation of intestinal bacteria is common in patients with chronic liver disease and causes characteristic infectious complications in advanced disease stages (Cirera et al., 2001; Schuppan and Afadh, 2008; Tandon and Garcia-Tsao, 2008). In addition, translocation of bacterial components termed pathogen-associated molecular patterns (PAMPs) triggers inflammatory responses through Toll-like receptors (TLRs) in both early and late disease stages (Dolganiuc et al., 2007; Fukui et al., 1991; Nolan and Leibowitz, 1978; Seki et al., 2007; Wiest and Garcia-Tsao, 2005). Moreover, PAMPs from the intestinal microbiota and TLR4 contribute to liver fibrosis and cirrhosis (Broitman et al., 1964; Rutenburg et al., 1957; Seki et al., 2007). Notably, 80% of hepatocellular carcinomas (HCCs) develop in fibrotic or cirrhotic livers as a consequence of chronic liver injury (Fattovich et al., 2004; Luedde and Schwabe, 2011), with HCC being the leading cause of death in patients with compensated liver cirrhosis (Fattovich et al., 2004). However, pathways that are responsible for the high rate of HCC development in the chronically injured and fibrotic liver remain largely unknown. Here, we hypothesize that inflammatory and fibrogenic...
responses induced by the gut microbiota and TLRs contribute to hepatocarcinogenesis and might represent targets for HCC prevention or treatment.

RESULTS

TLR4 Is Required for Hepatocarcinogenesis in the Chronically-Injured Liver

To determine whether the known contribution of TLRs to hepatic inflammation and fibrosis may set the stage for HCC development, we subjected C3H/HeJ and C3H/HeOuJ mice, which carry a nonfunctional and the wild-type Tlr4, respectively, to a combination of diethylnitrosamine (DEN) and the hepatotoxin carbon tetrachloride (CCl4). This model incorporates chronic injury, inflammation, fibrogenesis, and CCl4-mediated increases of endotoxin levels (Seki et al., 2007) and thus shares several features with the microenvironment in which the majority of human HCCs arise. Tumors developing by this regimen showed typical features of HCC, and microarray analysis revealed a characteristic HCC expression profile with Afp, Gpc3, and Cdkn2b among the most upregulated genes with greater than 90-fold increase in comparison to normal liver (data not shown). TLR4-wild-type (Tlr4WT) and TLR4-inactivated mutant (Tlr4mut) mice had a similar tumor incidence (100% in Tlr4WT mice and 78% in Tlr4mut mice, p = 0.2105), but Tlr4mut mice displayed a profound reduction of tumor number and size and an almost normal liver:body weight ratio 10.5 months after the initial DEN injection (Figure 1A).

Taking advantage of the separate initiation and promotion stages in this model, we further analyzed the contribution of TLR4 at these stages. Following DEN injection, we observed no differences in the induction of inflammatory genes Il6 and Ccl2 and p53-dependent genes Cdkn1a and Bax between Tlr4WT and Tlr4mut mice (Figure S1A available online). DEN-induced liver injury, as determined by serum ALT and AST levels, was exacerbated in Tlr4mut mice (Figure S1A). However, the increased liver injury is unlikely to cause decreased hepatocarcinogenesis in Tlr4mut mice, as liver injury after DEN is thought to promote hepatocarcinogenesis by inducing regenerative responses (Maeda et al., 2005; Naugler et al., 2007). Moreover, despite a small trend toward decreased HCC development, all Tlr4mut mice subjected to a purely genotoxic hepatocarcinogenesis protocol developed numerous large tumors (Figure S1B). Thus, TLR4 status exerts no major effect on genotoxic tumor initiation apart from an increased susceptibility of Tlr4mut mice to liver injury. In contrast, we observed a significant impact of TLR4 status on tumor promotion by CCl4, with proliferation markers Col1a1 and Acta2 and inflammatory markers Il6, Il1b, Tnf, and Ccl2 strongly reduced in Tlr4mut mice, as evidenced by microarray (data not shown) and qPCR analysis, despite increased liver inflammation, fibrogenesis, and CCl4-mediated increases of endotoxin levels (Seki et al., 2007) and thus shares several features with the microenvironment in which the majority of human HCCs arise. Tumors developing by this regimen showed
The Intestinal Microbiota, TLR4, and HCC

To exclude that our findings reflect a particular role for TLR4 in the response to CCl4, we tested two additional models in which DEN was combined with thioacetamide or a choline-deficient diet and found essentially the same results with decreased proliferation using Tlr4 knockout (Tlr4KO) mice in comparison to DEN plus CCl4-treated Tlr4WT mice in comparison to DEN plus CCl4-treated Tlr4WT mice. The right heatmap shows genes fulfilling the same conditions as described earlier restricted to the GO categories 0007049, 0009611, and 0005578. Data are represented as means ± SD. *p < 0.05. **p < 0.01. See also Figure S2.

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The Intestinal Microbiota and Lipopolysaccharide Promote Hepatocarcinogenesis

Next, we examined whether ligands from the bacterial intestinal microbiota are triggers for the observed TLR4-dependent tumor promotion. For this purpose, we gut-sterilized mice with a well-established cocktail of oral antibiotics that eliminates commensal bacteria (Rakoff-Nahoum et al., 2004) and reduces systemic lipopolysaccharide (LPS) levels (Seki et al., 2007). This treatment resulted in not only a >99.5% reduction of 16s levels systemic lipopolysaccharide (LPS) levels (Seki et al., 2007). This treatment resulted in not only a >99.5% reduction of 16s levels (Figure S1A) but also a highly significant reduction of tumor number, size, and the liver:body weight ratio in our DEN plus CCl4 HCC model of proliferation using Tlr4 knockout (Tlr4KO) mice in comparison to DEN plus CCl4-treated Tlr4WT mice in comparison to DEN plus CCl4-treated Tlr4WT mice. The right heatmap shows genes fulfilling the same conditions as described earlier restricted to the GO categories 0007049, 0009611, and 0005578. Data are represented as means ± SD. *p < 0.05. **p < 0.01. See also Figure S2.

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Similar to data in the Tlr4mut mice, tumor incidence was not affected by gut sterilization (100% in gut-sterilized vs. 100% in control mice). In addition to the similar degree of HCC reduction, gut sterilization markedly phenocopied events observed in the Tlr4mut mice such as reduced expression of cell cycle, fibrosis, and inflammatory genes and increased liver injury (Figure 2B; Figure S2B). Accordingly, the great majority of 1,752 genes that were significantly up- or downregulated by DEN plus CCl4 and significantly up- or downregulated by TLR4 inactivation were concordantly regulated in DEN plus CCl4 and significantly up- or downregulated by TLR4 inactivation and gut sterilization (Figure 2A). Similar to data in the Tlr4mut mice, tumor incidence observed in the Tlr4mut mice treated with subcutaneous PBS or LPS pumps for 2 weeks, or mice receiving antibiotics for 2 weeks. The chart contains a total of 118 taxa at the genus level, and the top 20 taxa are annotated on the left with most abundant taxa listed on top. Data are represented as means ± SD. *p < 0.05. **p < 0.01.

See also Figure S3.

Treatment led to a significant increase in inflammatory gene expression, tumor number, tumor size, and liver:body weight ratio (Figure 3A; Figure S3), further confirming our hypothesis that the LPS-TLR4 pathway promotes hepatocarcinogenesis. As Tlr5-deficient mice have an altered gut microbiome (Vijay-Kumar et al., 2010), it is conceivable that TLR4 might also affect the composition of the gut microbiome and thereby influence HCC development rather than exerting direct effects in the liver. To address this possibility, we first performed 16S-based pyrosequencing of the cecal gut microbiome in Tlr4WT and Tlr4mut mice, Tlr4WT mice treated with subcutaneous PBS or LPS pumps for 2 weeks, or mice receiving antibiotics for 2 weeks. The top 20 taxa are annotated on the left with most abundant taxa listed on top. Data are represented as means ± SD. *p < 0.05. **p < 0.01. See also Figure S3.
The intestinal microbiota, TLR4, and HCC

To determine the hepatic cell that promotes HCC in response to TLR4 activation, we generated TLR4-chimeric mice using a combination of irradiation, bone marrow transplantation (BMT), and liposomal clodronate-mediated macrophage depletion (Seki et al., 2009; Seki et al., 2007). This protocol achieved >97% suppression of LPS responses after transplantation of Tlr4mut bone marrow into Tlr4WT mice, almost complete restoration of LPS responses after transplantation of Tlr4WT bone marrow into Tlr4mut mice, and substitution of F4/80-positive hepatic macrophages (Figure S4A). DEN plus CCl4-treated Tlr4mut and gut-sterilized mice as well as from TLR4-chimeric mice with Tlr4mut in resident liver and Tlr4WT bone marrow (Figures 6A and 6C). Both HGF mRNA (data not shown) and epiregulin mRNA were expressed much higher in hepatic stellate cells than in whole liver, hepatocytes, or hepatic macrophages (Figure 6D). As the role of HGF in hepatocarcinogenesis remains controversial (Sakata et al., 1996; Santoni-Rugiu et al., 1996; Takami et al., 2007), we focused our attention on epiregulin. Hepatic stellate cells isolated from fibrotic livers secreted significantly more epiregulin than quiescent hepatic stellate cells (Figure 6D), and stimulation of hepatic stellate cells with LPS significantly upregulated epiregulin mRNA and protein in an NF-κB dependent manner (Figure 6E). Moreover, LPS injection into mice either as a bolus or via the aforementioned chronic subcutaneous infusion also significantly increased epiregulin expression in the liver (Figure 6D; Figure S3). Epiregulin mRNA expression was also induced by LPS in human hepatic stellate cells and upregulated in livers of patients with alcoholic hepatitis, a group of patients with typically very high LPS levels (Fukui et al., 1991) (Figure 6F).

To elucidate the contribution of epiregulin to hepatocarcinogenesis, we subjected wild-type and epiregulin-deficient EregKO mice to DEN plus CCl4-mediated hepatocarcinogenesis. Due to the much lower propensity of C57Bl/6 mice to develop HCC, we used an altered protocol in which mice were subjected to DEN at Day 15 postpartum, followed by multiple CCl4 injections. EregKO mice displayed a significant reduction of tumor number, size, and the liver:body weight ratio, albeit to a much lesser degree than Tlr4mut mice (Figure 6G). Moreover,
almost normalized the liver:body weight ratio (Figure 7A). At reduced tumor number and size by 90% and 70%, respectively, sterilization during the last 4 months of hepatocarcinogenesis (Figure 6H). Thus, additional factors must be responsible for the Tlr4mut striking reduction of hepatocarcinogenesis in Tlr4mutogenesis model and did not differ between wild-type, epiregulin was not upregulated at late stages in our hepatocarcinogenesis model and did not differ between wild-type, Tlr4mut, and gut-sterilized mice at these stages of hepatocarcinogenesis (Figure 6H). Thus, additional factors must be responsible for the striking reduction of hepatocarcinogenesis in Tlr4mut and gut-sterilized mice.

The Intestinal Microbiota and TLR4 Suppress Apoptosis in Late Stage of Hepatocarcinogenesis

Next, we attempted to establish the time frame during which bacterial PAMPs exert the most profound effects on hepatocarcinogenesis. Surprisingly, gut sterilization during the first 4.5 months of hepatocarcinogenesis showed only a moderate decrease of hepatocarcinogenesis (Figure 7A). In contrast, gut sterilization during the last 4 months of hepatocarcinogenesis reduced tumor number and size by 90% and 70%, respectively, and almost normalized the liver:body weight ratio (Figure 7A). At this point, mice had not yet developed macroscopically or microscopically visible tumors (Figure 7B), suggesting that gut sterilization prevented tumor development rather than leading to the regression of already established tumors. We additionally tested whether more selective gut decontamination with rifaximin, a widely used nonabsorbable antibiotic for the prevention of hepatic encephalopathy in patients with endstage liver disease (Bass et al., 2010), could influence hepatocarcinogenesis at late stages. We observed a borderline significant reduction of tumor numbers but saw no significant reduction in tumor size (data not shown), suggesting only moderate efficacy of rifaximin monotherapy on hepatocarcinogenesis at late stages (Figure S5).

To determine whether gut sterilization might affect already established tumors, we treated wild-type mice at a time point where tumors were already present with the quadruple antibiotics cocktail for 6 weeks. However, we found no significant change of tumor size, number, or liver:body weight ratio in comparison to control mice (Figure 7C). When following single tumors by weekly ultrasound monitoring, we also saw no change in tumor size in antibiotics-treated mice (data not shown).

To further determine the mechanism by which the intestinal microbiota and TLR4 promote hepatocarcinogenesis, we analyzed gene expression, proliferation, and apoptosis in gut-sterilized and Tlr4mut mice. Due to the lower tumor number this point, mice had not yet developed macroscopically or microscopically visible tumors (Figure 7B), suggesting that gut sterilization prevented tumor development rather than leading to the regression of already established tumors. We additionally tested whether more selective gut decontamination with rifaximin, a widely used nonabsorbable antibiotic for the prevention of hepatic encephalopathy in patients with endstage liver disease (Bass et al., 2010), could influence hepatocarcinogenesis at late stages. We observed a borderline significant reduction of tumor numbers but saw no significant reduction in tumor size (data not shown), suggesting only moderate efficacy of rifaximin monotherapy on hepatocarcinogenesis at late stages (Figure S5).

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To further determine the mechanism by which the intestinal microbiota and TLR4 promote hepatocarcinogenesis, we analyzed gene expression, proliferation, and apoptosis in gut-sterilized and Tlr4mut mice. Due to the lower tumor number
and size, the overall amount of Ki-67 staining in TLR4<sup>−/−</sup> and gut-sterilized mice was expectedly lower (data not shown). Microarray comparison showed a highly similar gene expression pattern in HCC from wild-type, TLR4<sup>−/−</sup>, and gut-sterilized mice (Figure S6A), and HCCs from these groups did not cluster separately as shown by dendrogram and Unifrac analysis (Figures S6B-S6C). There was a small but nonsignificant trend toward lower AFP and Ki-67 in HCCs from gut-sterilized mice and TLR4<sup>−/−</sup> mice, which was most likely due to the smaller size of the tumors in these groups. There were no genes that showed significant changes, as defined by a corrected p value of < 0.05, in the TLR4<sup>−/−</sup> or gut-sterilized HCC group when compared to the TLR4<sup>WT</sup> HCC group (data not shown). Moreover, there were no differences in tumor grade (Figure S6D), and there was a similar loss of collagen IV staining in HCCs from all groups (Figure S6E).

Microarray analysis showed no differences in expression of the cancer stem cell marker prominin 1/CD133 (data not shown), and we found only small and very rare clusters of cells positive for the progenitor marker A6 (Engelhardt et al., 1990), with mostly low A6 expression and morphology of intermediate hepatobiliary cells (Engelhardt et al., 1990), with mostly weak pan-cytokeratin expression typical of intermediate hepatobiliary cells in all groups (Figures S6G-S6H). Altogether, the microarray and immunohistochemical data suggest that TLR4 signaling is unlikely to promote hepatocarcinogenesis via progenitor cells. There was also no significant difference in Ki-67 positive cells in nontumor liver between these groups, suggesting that TLR4 and the intestinal microbiota do not

Figure 6. Epiregulin Is an LPS-Inducible Promoter of Hepatocarcinogenesis
(A) Ereg and Hgf mRNA were determined in TLR4<sup>−/−</sup> mice (n = 14), TLR4<sup>WT</sup> (n = 15) and antibiotics-treated TLR4<sup>WT</sup> (n = 9) after DEN and 2x CCl<sub>4</sub> injections. Fold induction versus untreated livers. (B) Ereg and Hgf mRNA were determined in TLR4-chimeric mice with TLR4<sup>WT</sup> resident cells and TLR4<sup>−/−</sup> bone marrow (n = 6) or TLR4<sup>−/−</sup> resident cells and TLR4<sup>WT</sup> bone marrow (n = 6) after treatment with DEN and two CCl<sub>4</sub> injections. Fold induction versus untreated livers.

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promote tumor formation by increasing proliferation in nontumor liver (Figure 8A).

Both gut-sterilized and Tlr4mut mice displayed a significant increase in cleaved-caspase 3-positive cells with predominant expression of cleaved-caspase 3 in hepatocytes in nontumor areas (Figure 8B). Notably, the number of cleaved caspase 3-positive cells was inversely and highly significantly correlated with tumor number, size, and the liver:body weight ratio (Figure 8B), further underlining the biological relevance of these findings. Significant increases in cleaved-caspase 3 positive cells and a strong and highly significant inverse correlation between cleaved-caspase 3 and tumor number, size, and liver:body weight ratio were also observed in mice that were gut-sterilized at late time points (Figures 8C and 8D). Moreover, the expression of NF-κB-regulated antiapoptotic genes Birc3, Birc5, and Nos2 (Hatano et al., 2001; Wang et al., 1998) was reduced in nontumor areas of livers from Tlr4mut and antibiotics-treated mice (Figure 8E), suggesting that TLR4 and the intestinal microbiota provide survival signals that are advantageous for hepatocarcinogenesis.

**DISCUSSION**

Chronic inflammation is a key contributor to carcinogenesis in various organs including the stomach, colon, lung, and liver (Balkwill and Mantovani, 2001; Grivennikov et al., 2010). In the liver, 80% of HCCs develop in a microenvironment characterized by chronic injury, inflammation, and fibrosis. However, mediators that are responsible for the high risk to develop HCC in the chronically injured liver are largely unknown. Translocation of bacteria and bacterial PAMPs is common in chronic liver disease (Schuppan and Afshal, 2008; Tandon and Garcia-Tsao, 2008) and promotes inflammation and fibrosis in chronic liver injury (Seki et al., 2007).

We now provide several lines of evidence that the intestinal microbiota and TLR4 link inflammation and carcinogenesis in the chronically injured liver. Genetic TLR4 inactivation, gut sterilization, or GF status decrease HCC development by ≈80%. Conversely, prolonged treatment with low-dose LPS significantly increases HCC development. These findings are similar to intestinal tumorigenesis in which TLR4 and MyD88 drive carcinogenesis (Fukata et al., 2007; Lee et al., 2010; Rakoff-Nahoum and Medzhitov, 2007). In contrast to the intestine, the liver is not in direct contact with intestinal bacteria. However, the liver is the first target of intestinal bacteria and PAMPs translocating into the portal circulation. Moreover, translocation increases with the development of liver fibrosis and cirrhosis (Fukui et al., 1991; Lin et al., 1995; Wiest and Garcia-Tsao, 2005). Our HCC data in GF mice effectively exclude the possibility that the employed antibiotics with low systemic bioavailability might have exerted direct effects on the liver in our model. Moreover, 16s pyrosequencing demonstrated that TLR4 status and LPS treatment do not significantly change...
the composition of the gut microbiome, with the exception of one taxon whose abundance differed between LPS- and PBS-treated mice but not between Tlr4WT and Tlr4mut mice.

In contrast to a previous study (Yu et al., 2010), we find no significant contribution of the intestinal microbiota and TLR4 to tumor initiation or purely genotoxic hepatocarcinogenesis but demonstrate two distinct mechanisms through which the intestinal microbiota and TLR4 promote HCC: In early stages, TLR4 deficiency and gut sterilization decrease hepatic proliferation and fibrogenesis. Accordingly, gut sterilization or LPS infusion given during early stages moderately decrease or increase hepatocarcinogenesis, respectively. Our study provides evidence that TLR4-dependent HCC promotion in the early phases is predominantly mediated by TLR4-dependent secretion of growth factors such as epiregulin by hepatic stellate cells. Thus, hepatic stellate cells might provide a link between fibrosis and HCC development by increasing growth factors. Based on previous studies showing promotion of growth factor signaling and tumor development by ECM-rich and stiff environment (Levental et al., 2009; Samuel et al., 2011), it is likely that growth factors and ECM produced by hepatic stellate cells synergize in tumor promotion. Our results are consistent with recent studies showing TLR4- and MyD88/Trif-dependent regulation of epiregulin and regeneration after injury in the intestine (Brandl et al., 2010; Hsu et al., 2010). However, HCC reduction in epiregulin-deficient mice was less pronounced than in Tlr4mut or gut-sterilized mice, suggesting that additional mechanisms contribute to TLR4-dependent HCC promotion.

The most significant contribution of the intestinal microbiota in our study occurred during later stages of hepatocarcinogenesis independently of CCl4-induced upregulation of growth factors and regenerative responses. These results are remarkably similar to a previous study in which NF-B inhibition at late, but not early, stages reduced inflammation-associated HCC (Pikarsky et al., 2004). In line with this study, we detected decreased induction of NF-B regulated genes and an increased rate of apoptosis in all groups that were protected from HCC development. Accordingly, we found that the hepatocyte compartment which had been unresponsive to LPS in healthy livers became highly sensitive to LPS in DEN plus CCl4-injured livers. The biological relevance of increased apoptosis for HCC development is emphasized by the strong inverse correlation of tumor number, tumor size, and liver:body weight ratio with cleaved-caspase 3-positive cells in two sets of gut-sterilized mice and in Tlr4mut mice. In addition to its role in Abcb4KO chronic liver

Figure 8. TLR4 and the Intestinal Microbiota Protect from Apoptosis

(A–B) Livers from Tlr4WT (n = 11), Tlr4mut (n = 9) or antibiotics-treated (Abx) Tlr4mut mice (n = 7) were stained for Ki-67 (A) or cleaved-caspase 3 (B) followed by quantification. Correlation between the number of cleaved-caspase 3 positive cells and tumor number, size and liver:body weight ratio was determined.

(C–D) Tlr4WT were either not gut sterilized (n = 7) or gut sterilized 2 weeks after the last CCl4 injection until they were sacrificed (n = 8). Livers were stained for Ki-67 and cleaved-caspase 3 followed by quantification (C). Correlation between the number of cleaved-caspase 3 positive cells and tumor number, size, and liver:body weight ratio was determined (D).

(E) Nos2, Birc3, and Birc5 mRNA levels were compared between livers from Tlr4WT (n = 12) and Tlr4mut (n = 9) mice and between untreated wild-type mice (WT, n = 9) and antibiotics-treated wild-type mice (Abx, n = 6) Tlr4WT mice. Data are represented as means ± SD. *p < 0.05. See also Figure S6.
injury model (Pikarsky et al., 2004), hepatocellular NF-κB also acts as a promoter of hepatocarcinogenesis in lymphotoxin-driven HCC (Haybaeck et al., 2008), suggesting a requirement for hepatocellular NF-κB in HCC occurring in the setting of significant inflammation. In contrast, hepatocellular inactivation of NF-κB in genotoxic HCC models enhances hepatocarcinogenesis (He et al., 2010; Maeda et al., 2005). Finally, the complete but rather unphysiologically absent of NF-κB activity by Nemo or TAK1 deletion causes chronic hepatocyte injury, liver cirrhosis, and spontaneous HCC (Bettermann et al., 2010; Inokuchi et al., 2010; Luedde et al., 2007). Thus, the presence of inflammation and the degree of NF-κB inhibition appear to be crucial factors that determine whether NF-κB promotes or inhibits hepatocarcinogenesis.

The majority of apoptosis in our study occurred in nontumor areas of the liver suggesting that prosurvival signals provided by the intestinal microbiota and TLR4 are required for the survival of tumor precursors and that apoptosis prevents the development of tumor lesions rather than leading to regression of already established tumors. Accordingly, we observed no effect of gut sterilization on already established HCCs. These results are consistent with previous findings that gene expression profiles in nontumor liver tissue, including gene sets related to NF-κB, TNFα, and interferon signaling—all downstream targets of TLR4—are crucial determinants of tumor recurrence, whereas tumor profiles have no prognostic value (Hoshida et al., 2008).

One important difference between our findings and many previous studies is the tumor-promoting effect of inflammatory signals in resident liver cells. Bone-marrow-derived cells such as macrophages are abundant sources of inflammatory cytokines and contribute to genotoxic HCC (Maeda et al., 2005). Our studies in chimeric mice demonstrate that bone-marrow-derived cells including hepatic macrophages do not mediate the tumor-promoting effects of TLR4 in both acute and long-term HCC models. Accordingly, we found that both hepatic stellate cells and hepatocytes are targets of LPS in the liver with TLR4 on hepatic stellate cells most likely mediating regenerative response after injury and TLR4 on hepatocytes preventing apoptosis of hepatocytes and premalignant cells. Oval cells represent another potential target of LPS and LPS-induced mediators such as TNFα (Brooling et al., 2005), but we found only a few areas with A6- and cytokeratin-positive cells typical of oval cells in our tumor model and no influence of TLR4 status and gut sterilization on the number of these cells. Further studies in mice with conditional TLR4 deletion are required to distinguish the relative contribution of TLR4 expressed in hepatocytes versus TLR4 expressed in hepatic stellate cells to hepatocarcinogenesis.

Together with results from a previous study (Pikarsky et al., 2004), our data suggest that chronic liver injury affects the resident liver compartment and makes resident liver cells and/or tumor-forming cells more dependent on inflammatory signals for their survival. With the majority of HCCs occurring in chronically injured livers but many mouse models lacking chronic injury, it is possible that the contribution of inflammatory pathway to human HCC has been underestimated. Our results differ from a previous study in which bone-marrow-derived cell populations were suggested to promote HCC through TLR4 (Yu et al., 2010). Notably, conclusions on the contribution of bone marrow were based on acute responses to DEN instead of HCC development in that study. Moreover, TLR4- and MyD88-dependent carcinogenesis in the intestine and hepatic carcinogenesis in HCV transgenic mice are also promoted by resident cells (Hsu et al., 2010; Lee et al., 2010; Machida et al., 2009; Rakoff-Nahoum and Medzhitov, 2007). In the latter study, TLR4 promotes HCC in alcohol-treated HCV transgenic mice through the upregulation of stem cell markers. Although we observed a strong increase in the stem cell marker prominin 1/CD133 in HCCs, we observed no influence of TLR4 status on its expression in our microarray analysis, suggesting that there may be additional mechanisms by which TLR4 promotes hepatocarcinogenesis in alcohol-treated HCV transgenic mice.

To enable bone marrow transplantation studies in adult mice, our study took advantage of the high HCC susceptibility of the C3H strain and used Tlr4WT C3H/HeOuJ and Tlr4mut C3H/HeJ mice. We can exclude that genetic differences besides TLR4 play a role in the observed promotion of HCC by TLR4 and the intestinal microbiota based on the following findings: (a) The Tlr4 locus is the only SNP among 2,059 tested SNPs that differs between C3H/HeOuJ and C3H/HeJ mice (www.jax.org/phenome). (b) Findings in C3H/HeOuJ and C3H/HeJ mice were confirmed in wild-type and Tlr4KO C57B/6 mice. (c) A large set of experiments solely performed in C3H/HeOuJ mice such as gut sterilization and LPS infusion by osmotic pumps demonstrated the HCC-promoting role for the intestinal microbiota and the TLR4 ligand LPS.

The presented data provide conclusive evidence that the intestinal microbiota exerts a profound influence on HCC promotion in the chronically injured liver. Moreover, our study provides a strong rationale for targeting the intestinal microbiota and TLR4 for the primary or secondary prevention of HCC. The astounding reduction of HCC by gut sterilization at late stages suggests that these treatments might exert protective effects even in advanced liver disease. HCC has become the major cause of death in patients with compensated liver cirrhosis, and there is a need for more effective treatment and preventative strategies (Bruix et al., 2004). To translate our findings into clinical medicine, clinically feasible methods of targeting the intestinal microbiota or TLR4 need to be established. The quadruple combination of antibiotics used in the present study almost completely decontaminated the intestine and prevented 80% of murine HCCs but is not suitable for long-term treatment due to known side effects in patients with advanced liver disease. Our study suggests that rifaximin, a nonabsorbable antibiotic that is widely used in patients with advanced liver disease, may moderately reduce HCC development. Further studies are required to determine if longer treatment duration or combination with other well-tolerated antibiotics could improve the effects of rifaximin or if other approaches such as TLR4 antagonists would reduce HCC development.

EXPERIMENTAL PROCEDURES

Mice: HCC Induction and Evaluation
C3H/HeOuJ, C3H/HeJ, TLR2-deficient mice, TLR4-deficient mice (C57Bl6/10ScJ), TNFR1/-/-1R1/-/double deficient, and C57Bl/6 mice were purchased.
from Jackson Laboratories. Ereg-deficient mice in a C57Bl/6 background were obtained from the Mouse Mutant Regional Resource Center at the University of North Carolina.

In C3H/HeOuJ and C3H/HeJ mice, HCC was induced by intraperitoneal (i.p.) injection of DEN (100 mg/kg) at ages 6-14 weeks followed by 6-12 biweekly injections of carbon tetrachloride (0.5 mg/kg i.p., dissolved in corn oil) unless stated otherwise. For Ereg-deficient mice and C57Bl/6 wild-type controls, HCC was induced by the combination of DEN (25 mg/kg i.p.) given at Day 15 postpartum and 22 weekly injections of CCl₄ (0.5 mg/kg i.p., dissolved in corn oil). For Tlr4⁻/⁻ mice and C57Bl/6 wild-type controls, HCC was induced by the combination of DEN (100 mg/kg, i.p.) given at the age of 8 weeks, followed by 25 biweekly injections of CCl₄ (0.5 mg/kg, i.p., dissolved in corn oil). Gut sterilization was done as previously described (Rakoff-Nahmou et al., 2004; Seki et al., 2007) using a combination of ampicillin (1 g/l), neomycin (1 g/l), metronidazole (1 g/l), and vancomycin (500 mg/l) in drinking water. For rifaximin studies, mice received either 250 mg/kg rifaximin in drinking water with rifaximin dissolved in Ora-Plus/Ora-Sweet (Cober et al., 2010) or Ora-Plus/Ora-Sweet vehicle translating liver metastases (n = 2). All animals were sacrificed by cervical dislocation and hearts were removed for further studies.

Evaluation of tumor number and size was determined as described by counting the number of visible tumors and measuring the size of the largest tumor with a caliper (Naugler et al., 2007). After sacrifice, livers were explanted, digitally photographed, and weighed to calculate the liver:body weight ratio. To evaluate acute effects of TLR4 and the intestinal microbiota, adult mice were treated with DEN (100 mg/kg i.p.) alone or in combination with either two or four injections of CCl₄ (0.5 mg/kg i.p.), two injections of thioacetamide (400 mg/kg i.p.) or 4 weeks of a choline-deficient L-amino-acid-defined diet. For some experiments, mice were treated with lipid-based vehicles (200 μl i.p.) or 4 weeks of a choline-deficient L-amino-acid-defined diet. For some experiments, liver fibrosis was induced by ligating the common bile duct as previously described (Seki et al., 2007; Kluwe et al., 2010) and used between passages 3 and 5. Procedures involving human materials were performed using a transjugular approach. In all patients, liver specimens were prospectively obtained in the Liver Unit of the Hospital Clínic, Barcelona, between January 2009 and December 2010 (n = 10). Liver biopsies were obtained using a transjugular approach. In all patients, liver specimens were analyzed by an expert liver pathologist. Fragments of normal livers were obtained from optimal cadaveric liver donors (n = 2) or resection of liver metastases (n = 2). All control and normal serum samples were transferrase at the University of North Carolina National Gnotobiotic Rodent Resource Center, Chapel Hill, NC. All mice received DEN (25 mg/kg i.p.) in the GF status at Day 15 postpartum. One week after DEN, 50% of mice were moved to an SPF environment. Four weeks after the DEN injection, GF and SPF mice received a total of 10 CCl₄ injections (1 injection per week, at 0.5 ml/kg i.p. in corn oil). GF status was confirmed by biweekly monitoring for the presence of bacteria in the feces by aerobic and anaerobic culture as well as Gram staining of the stool. Mice were sacrificed 30 weeks after the initial DEN injection.

Human Samples and Cells

Patients with clinical, analytical, and histological features of alcoholic hepatitis were prospectively obtained in the Liver Unit of the Hospital Clinic, Barcelona, between January 2009 and December 2010 (n = 10). Liver biopsies were obtained using a transjugular approach. In all patients, liver specimens were analyzed by an expert liver pathologist. Fragments of normal livers were obtained from optimal cadaveric liver donors (n = 2) or resection of liver metastases (n = 2). All control and normal serum samples were transferrase at the University of North Carolina National Gnotobiotic Rodent Resource Center, Chapel Hill, NC. All mice received DEN (25 mg/kg i.p.) in the GF status at Day 15 postpartum. One week after DEN, 50% of mice were moved to an SPF environment. Four weeks after the DEN injection, GF and SPF mice received a total of 10 CCl₄ injections (1 injection per week, at 0.5 ml/kg i.p. in corn oil). GF status was confirmed by biweekly monitoring for the presence of bacteria in the feces by aerobic and anaerobic culture as well as Gram staining of the stool. Mice were sacrificed 30 weeks after the initial DEN injection.

Primary Cell Isolations

Primary hepatic stellate cells, Kupffer cells, and hepatocytes were isolated from either normal or DEN plus 2x CCl₄-treated mice using retrograde liver perfusion and isolation techniques as previously described (Seki et al., 2007). For some experiments, hepatic stellate cells were infected with an adenosine expressing IxB superrepressor (AdIxB) or an empty control adenosine as described elsewhere (Seki et al., 2007).


