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Alcohol, TLR4-TGF-β Antagonism, and Liver Cancer

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Abstract

Alcohol abuse and obesity are two known risk factors for hepatocellular carcinoma (HCC) that also synergistically promote HBV/HCV-related carcinogenesis. TLR4, the PAMP for endotoxin participates in inflammatory processes such as M1 activation of hepatic macrophages in alcoholic liver disease. However its role in liver carcinogenesis via ectopic expression and activation, has only recently been revealed in alcohol/HCV-associated HCC models. Alcohol feeding to mice expressing the HCV *Ns5a* in a hepatocyte specific manner, aggravates liver inflammation via activation of overexpressed TLR4 in the parenchymal cells. Long-term alcohol feeding produces liver tumors in these transgenic mice in a manner dependent on TLR4. From these mice, CD133+/CD49f+ tumor initiating stem cell-like cells (TICs) have been isolated. These TICs exhibit self-renewal and tumorigenic activities driven by TLR4-dependent upregulation of the stem cell factor NANOG. Defective TGF-β tumor suppressor pathway is identified in the TICs and mediated by NANOG target genes *Igf2bp3* and *Yap1*. This TGF-β pathway antagonism is responsible in part for TIC's tumorigenic activity and chemoresistance. Conversely, mice with attenuated TGF-β pathway due to haploinsufficiency of β2-Spectrin, spontaneously develop liver tumors and alcohol-feeding increases tumor incidence in a TLR4 dependent manner. This reciprocal antagonism between TLR4 and TGF-β pathways may serve as a novel therapeutic target for HCC.

Keywords

TLR4; cancer stem cells; NANOG; TGF-β

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Compliance with Ethical Requirements

The studies were also conducted following full approval of IRB and IACUC protocols by respective institutions for appropriate involvement of human and animal subjects.

Hidekazu Tsukamoto, Lopa Mishra, and Keigo Machida declare no conflict of interest.

Liver cancer epidemiology: importance of synergism among alcohol, obesity and HCV

Hepatocellular carcinoma (HCC) is the most prevalent malignancy of the liver and the 5th most common cancer in men. HCC is diagnosed in over half a million patients world-wide annually and is the second leading cause of the cancer-related death. Cirrhosis is the far most important risk factor for HCC, increasing the risk by 40-fold compared to normal subjects and ~70% of HCC patients have underlying cirrhosis. Despite this tight association, molecular mechanisms by which cirrhosis promotes HCC risk are still elusive. Viral hepatitis (HBV and HCV) is the most common etiological factor for HCC, followed by alcoholic liver disease (ALD). In particular, chronic infection with HCV represents a major risk for HCC (1) as more than 170 million people are infected with HCV globally (1–3). HCV produces proteins which are directly implicated in hepatocyte signaling or metabolic dysregulation, toxicity, and transformation. For instance, the HCV Core protein stimulates production of reactive oxygen species which can cause mitochondrial or nuclear DNA damage (2, 4, 5). The Core protein also inhibits microsomal triglyceride transfer protein and VLDL secretion (6), contributing to the genesis of fatty liver. The Core also induces insulin resistance *in vitro* and *in vivo*, and this effect may be mediated by degradation of insulin receptor substrates (IRS) 1 and 2 via upregulation of SOCS3 (7) and IRS serine phosphorylation (8). The latter mechanisms are also relevant to another disease which is increasingly recognized to promote HCC risk: non-alcoholic fatty liver disease (NAFLD) (9) that is a liver phenotype of obesity-associated metabolic syndrome. In fact, HCV/HBV infection, ALD, and NAFLD share common pathophysiological characteristics such as oxidant stress, organelle stress, and metabolic dysregulation which individually or collectively contribute to their oncogenic activities. More importantly, a striking synergism among HCV, alcohol, and obesity exists for the risk of HCC. Existence of co-morbidities such as alcohol abuse or obesity, increases the HCV risk of developing HCC by additional 8 fold, resulting in an overall 45–55 fold increase in the risk as compared to subjects without HCV infection and the co-morbidities (10, 11). As alcohol and obesity continue to dominate as leading life-style factors for disease burden around the world (12), heightened HCC incidence caused by synergistic interactions by these factors and hepatitis virus, represents the most predictable and devastating global health issue.

Inflammation and cancer

Since Rudolf Virchow noted leukocytes in tumor tissues 150 years ago, a link between inflammation and cancer has been an important topic in cancer research (13, 14). Approximately 15–20% of tumor cases are causally associated with infection (15) and chronic inflammation precedes and accompanies tumor development induced by many of tumor causing agents including chemical and physical agents, infectious agents, and autoimmune reactions. Best examples of malignancies caused by infection include gastric cancer caused by *Helicobacter pylori*, bladder and colon carcinoma caused by schistosomiasis, and HCC caused by HCV and HBV. Further the roles of sterile inflammation in tumorigenesis are also increasingly recognized. Experimental evidence

generated during the last two decades implicates the involvement of many inflammatory mediators (e.g., TNF-α, CXCL1, CXCR2, MIF, IKK) in the promotion of various tumors. For instance, macrophage migration inhibitory factor (MIF) released by T lymphocytes and macrophages, is shown to suppress transcriptional activity of p53 to promote tumor formation (16). However, whether and how inflammatory pathways initiate tumorigenesis are still elusive other than a very descriptive concept of DNA damage caused by oxygen and nitrogen radical species produced by infiltrating inflammatory cells. IκB kinase (IKK) which phosphorylates IκB for its degradation and consequent nuclear translocation of active p65/p50, is the most critical mediator of inflammation and cell survival. Deficiency of IKKβ, the catalytic subunit, in hepatocytes predisposes the liver for diethylnitrasamine (DEN)-mediated hepatocarcinogenesis via enhanced hepatocellular death and compensatory proliferation due to sustained JNK activation (17). On the other hand, myeloid specific *Ikkb* knockout attenuates liver cancer incidence due in part to suppression of MyD88-dependent IL-6 production which also appears to determine the gender disparity in liver cancer development (18). Although this study highlights the role of the macrophage-derived cytokine, cytokines and chemokines released from tumor cells or cells subjected to transformation insults, may also play a role as shown for production of CXCR2-activating chemokines by primary intestinal adenomas and other tumors (19). This study also demonstrates the importance of CXCR2 on Ly6G+ neutrophils for neutrophil recruitment to tumor sites and promotion of tumor growth and expansion. In Ly6G+ depleted mice, these CXCR2 mediated effects are abrogated and tumor cells even undergo apoptosis (19). A similar notion linking neutrophils and HCC needs to be investigated particularly addressing the relationship between alcoholic hepatitis and HCC development.

TLR4 links inflammation to tumorigenesis

The pathogenetic roles of TLR4 and its ligand endotoxin in alcoholic liver disease (ALD) have been established (20–22). Here endotoxin that enters the portal circulation due to alcohol-induced gut permeability, activates TLR4 on hepatic macrophages to stimulate the release of proinflammatory cytokines such as TNF-α and to exert cytotoxic effects on hepatocytes which are sensitized by alcohol (23). Interestingly, TLR4 is ectopically upregulated in hepatocytes in HCV *Ns5a* transgenic (Tg) mice (24). When these mice are challenged by intraperitoneal injection of a sublethal dose of LPS, fulminant hepatitis with marked increases in plasma AST and TNFα and mortality ensues, but not in wild type (WT) or *Ns5a Tg:Tlr4−/−* compound mice (24). Similarly, intragastric feeding of ethanol and high fat liquid diet to these Tg mice results in more severe alcoholic steatohepatitis compared to WT mice despite similar endotoxemia, and some mice even develop hepatitis with mid-zonal necrosis and mononuclear and neutrophilic infiltration (24). As expected, this aggravating effect is prevented in *Ns5a Tg:Tlr4−/−* mice, incriminating the role of ectopically induced hepatocyte TLR4 in accentuating liver inflammation. When Polymixin B and Neomycin are administered to alcohol-fed *Ns5a Tg* mice, liver injury is attenuated, and enteral administration of LPS to the mice worsens liver injury, supporting the role of gut endotoxin (24). When *Ns5a Tg* mice are fed alcohol liquid diet *ad libitum* for 12 months, liver tumor develops in 23% of mice while no tumor incidence is noted in alcohol-fed WT or *Ns5aTg:Tlr4−/−* mice deficient in TLR4 (24). These results suggest activation of ectopically

upregulated TLR4 by alcohol-induced endotoxemia, potentiates alcoholic liver inflammation in the short-term and induces liver tumor in the long-term, supporting an unequivocal role of TLR4 in linking inflammation to tumorgenesis in this model (Figure 1). A subsequent study has extended this observation to HCV *Core* Tg mice, basically confirming the role of TLR4 in alcohol-induced liver carcinogenesis (25).

In fact, TLR4 is already implicated in lung (26), colon (27), and skin carcinomas (28). Although we consider macrophages and lymphocytes are the primary cell types which express TLR4, increasing evidence points to the role of TLR4 ectopically expressed in epithelial parenchymal cells in oncogenesis (24, 29). A recent study from Robert Schwabe's laboratory demonstrates HCC development in mice induced by DEN and carbon tetrachloride requires the intestinal microbiota and TLR activation in non-bone marrow-derived resident liver cells (30), suggesting the importance of TLR4 on hepatocytes, biliary epithelial cells, and/or hepatic stellate cells. Our aforementioned results point to the role of hepatocytes in hepatocarcinogenesis although this has to be confirmed in other non-HCV models. This study from Schwabe's laboratory also concludes that TLR4 and the intestinal microbiota are required for HCC promotion but not HCC initiation (30). In contrast, our studies on alcohol-fed HCV Tg mice and tumor initiating stem cell-like cells (TICs) isolated from these models demonstrate TLR4 activation induces the key pluripotency factor NANOG and other stem cell factors required for the generation of CD133+/CD49f+ TICs and the development of liver tumors (25).

The involvement of inflammasomes in liver inflammation is increasingly recognized (31) where endogenous damaged-associated molecular patterns (DAMP) triggers sterile inflammation via the assembly of the inflammasome and consequent activation of caspase-1, interleukin-1 and other cytokines. Indeed, recent studies demonstrate the involvement of this inflammatory pathway in alcoholic steatohepatitis in mice (32) and hepatitis associated with HCV infection (33). Whether this pathway also links inflammation to liver oncogenesis, is yet to be tested.

The most recent study has identified and isolated liver cancer progenitors from the pre-malignant lesions in different mouse HCC models, and these cells are shown to give rise to liver cancer when introduced into a liver undergoing chronic damage and compensatory proliferation (34). These cells reside within dysplastic lesions and are suggested to arise from dysplastic hepatocytes and form tumors via autocrine IL-6 signaling. It is yet to be determined whether our TLR4-dependent TICs are traced back to dysplastic cells and whether the cancer progenitors from this study are also driven by the TLR4 pathway.

TLR4-deopendent TICs and TLR4-TGF-β antagonism

Immunostaining of liver tumor sections from alcohol-fed *Ns5a* Tg mice reveals small cells with an increased nuclear to cytoplasmic ratio positively stained for both NANOG and CD133, the marker for progenitor cells (24). This prompted us to isolate CD133-positive cells via FACS. Using this approach, a very small yet significantly increased percentage of CD133+/CD49f+ cells is isolated from liver tumors of alcohol-fed *Ns5a* Tg mice compared to normal livers of WT mice (1.11% vs. 0.05%) (25). Analysis of the CD133+/CD49f+ cells

show enhanced expression of stemness genes such as *Nanog, Oct4, Sox2* as compared to CD133−/Cd49f+ cells, and these inductions are abrogated by knockdown of *Tlr4*. Further, the cells grow anchorage independently in soft agar and form spheroids in methylcellulose culture in a manner dependent on TLR4 and NANOG (25). Finally, the tumor-initiating property of CD133+/CD49f+ cells is confirmed by tumor development by serial transplantation in NOG mice. As predicted, this activity is also TLR4 and NANOG dependent. Of interest, these TLR4/NANOG-dependent TICs are defective in the TGF-β tumor suppressor pathway because NANOG induces IGF2BP3 and YAP1 which block the TGF-β pathway at the level of SMAD3 phosphor-activation and p-SMAD3 nuclear translocation, respectively (25). Further, IGF2BP3-mediated AKT activation phosphorylates YAP1 to enhance YAP1's ability to retain SMAD in the cytosol. Knockdown of these two proteins restores TGF-β pathway, suppresses self-renewal and tumorigenic activity, and enhances chemosensitivity in TICs. Conversely, reduced TGF-β tumor suppressor pathway caused by haploinsufficiency of β2-Spectrin, the chaperon protein required for p-SMAD nuclear translocation, results in spontaneous liver tumor development in a manner dependent on ectopically induced and activated TLR4, and this effect is aggravated by alcohol intake. Knockdown of β2-Spectrin in Huh7 cells significantly increases TLR4 expression, and the cells' self-renewal and tumorigenic activities. The latter effects are abrogated by concomitant knockdown of TLR4, extending the relevance of the TLR4-TGF-β antagonism to humans. In essence, tipping the balance of the reciprocal antagonism between TLR4 oncogenic and TGF-β tumor suppressor pathways, appears to determine whether tumor initiating occurs, and this notion signifies the importance of this antagonism as a novel potential therapeutic target for HCC (see a schematic diagram shown in Figure 2).

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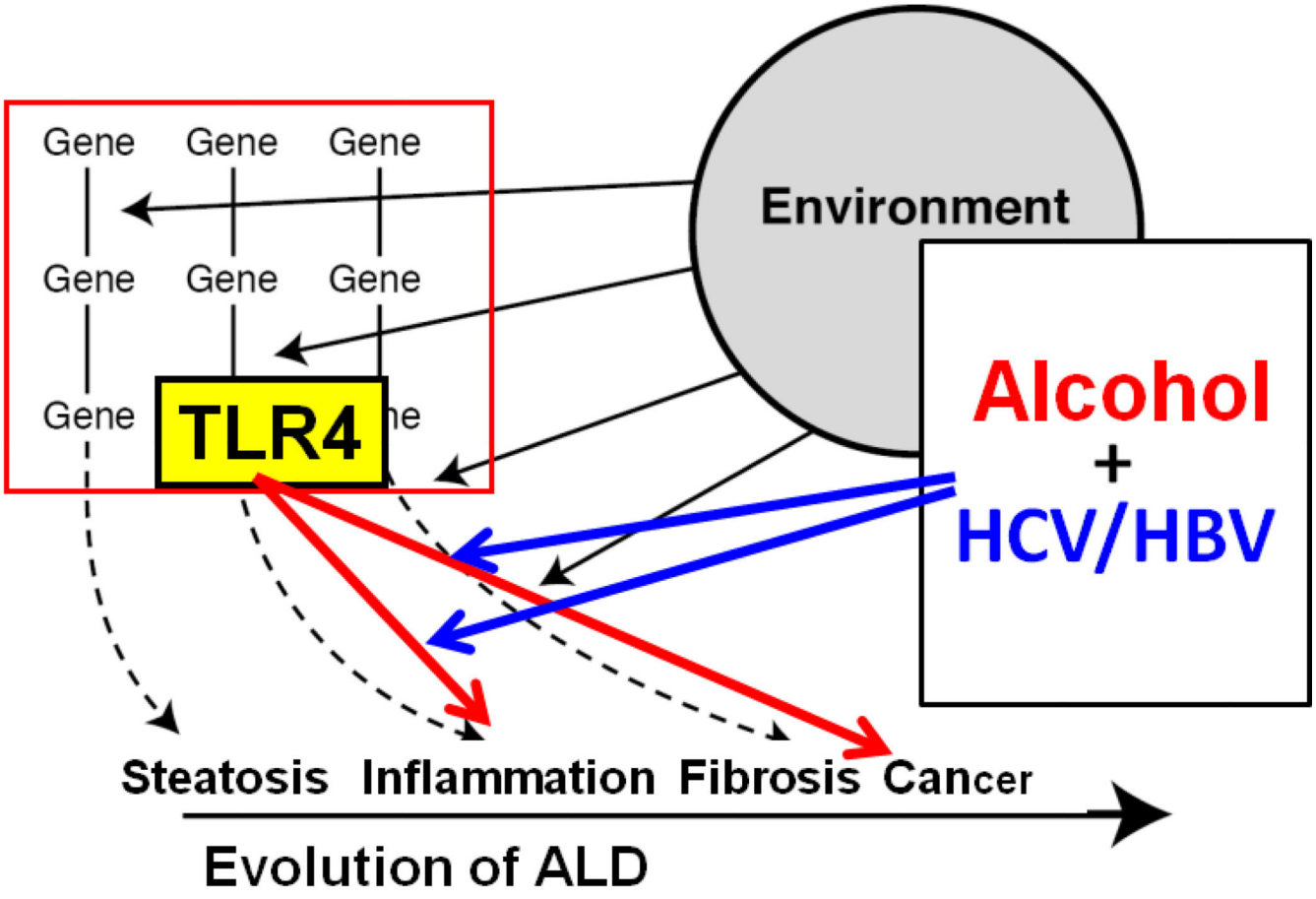

Figure 1.

This schematic diagram depicts that alcohol and HCV interact to render synergistic effects on the evolution of alcoholic liver disease that is governed by gene-environment interactions (dotted arrows and solid black lines). TLR4 mediates promotion of liver inflammation and cancer as depicted by red arrows and alcohol and HCV facilitate these TLR4-dependent mechanisms as shown by blue arrows.

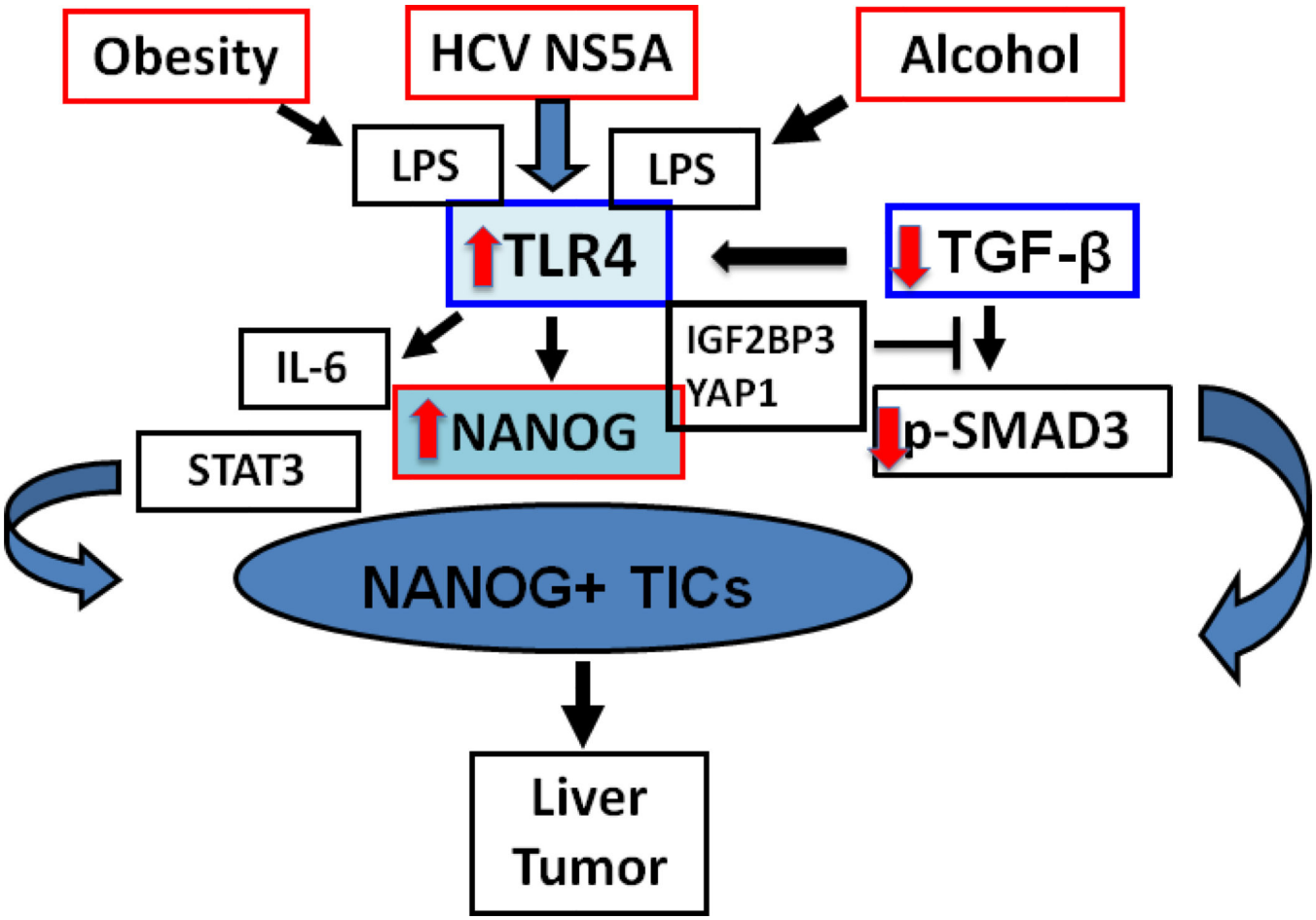

Figure 2.

Reciprocal antagonism by TLR4 oncogenic and TGF-β tumor suppressor pathways. TLR4 is ectopically upregulated in hepatocytes by HCV NS5A and activated by endotoxemia caused by alcohol intake or obesity. Activated TLR4 induces the pluripotency factor NANOG to generate tumor-initiating stem cell-like cells (TICs) partly via compromised TGF-β pathway by IGF2BP3 and YAP1 induced by NANOG. Conversely, defective TGF-β pathway upregulates TLR4 and causes liver tumorigenesis.