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Inflammatory models drastically alter tumor growth and the immune microenvironment in hepatocellular carcinoma

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Abstract

Initiation and progression of hepatocellular carcinoma (HCC) is intimately associated with a chronically diseased liver tissue. This diseased liver tissue background is a drastically different microenvironment from the healthy liver, especially with regard to immune cell prevalence and presence of mediators of immune function. To better understand the consequences of liver disease on tumor growth and the interplay with its microenvironment, we utilized two standard methods of fibrosis induction and orthotopic implantation of tumors into the inflamed and fibrotic liver to mimic the liver condition in human HCC patients. Compared to non-diseased controls, tumor growth was significantly enhanced under fibrotic conditions. The immune cells that infiltrated the tumors were also drastically different, with decreased numbers of natural killer cells but greatly increased numbers of immune-suppressive CD11b⁺ Gr1^{hi} myeloid cells in both models of fibrosis. In addition, there were model-specific differences: Increased numbers of CD11b⁺ myeloid cells and CD4⁺ CD25⁺ T cells were found in tumors in the bile duct ligation model but not in the carbon tetrachloride model. Induction of fibrosis altered the cytokine production of implanted tumor cells, which could have farreaching consequences on the immune infiltrate and its functionality. Taken together, this work demonstrates that the combination of fibrosis induction with orthotopic tumor implantation results in a markedly different tumor microenvironment and

tumor growth kinetics, emphasizing the necessity for more accurate modeling of HCC progression in mice, which takes into account the drastic changes in the tissue caused by chronic liver disease.

Keywords

Hepatocellular carcinoma; Fibrosis; Tumor microenvironment; Carbon tetrachloride; Bile duct ligation; Cytokines

1 Introduction

Liver cancer is the fifth most common cancer in men, seventh most common cancer in women, and the third most deadly cancer worldwide [1, 2]. Liver cancer, particularly hepatocellular carcinoma (HCC), almost always develops on the background of a chronically diseased liver tissue, induced by long-term exposure to an inflammatory stimulus, such as hepatitis viral infections, excessive alcohol consumption, or metabolic syndrome [1, 3]. These stimuli lead to the induction of hepatocyte death and compensatory proliferation, hepatic stellate cell activation, and immune infiltration [3]. This process frequently results in a fibrotic and ultimately cirrhotic liver microenvironment; in this context, the cycling hepatocytes accumulate genetic alterations and eventually undergo malignant transformation [3]. This is in stark contrast with many other cancer types, in which the tissue from which the tumor arises is generally quite normal and functional [4, 5]. Indeed, 80 %–90 % of patients, whom have developed liver cancer after a long history of liver illness, have cirrhosis of the liver or late-stage fibrosis [2]. The fibrotic liver, with its characteristic scarring of the tissue, comes with a concurrent production of numerous cytokines, chemokines, and growth factors, all of which shape the microenvironment and its constituents during hepatocarcinogenesis and progression [6–9]. With this common theme of inflammation and fibrosis accompanying and driving malignant transformation, a better understanding of the microenvironment and its effects on tumor progression can yield valuable insights into these processes as well as produce potential targets for therapeutic intervention.

To investigate the development and progression of HCC, mouse models are essential tools to dissect the complex roles of tumor cells and stromal components in the tumor microenvironment. Although numerous mouse models have been created in which liver cancer is induced either by carcinogens or by transgenic activation of oncogenes combined with inactivated tumor suppressor genes, it has been difficult to mimic the pathological process of the most common HCC types, those initiated by hepatitis viral infections. Thus, orthotopic transplantation of cancer cells, via direct injection into the liver or inoculation into the spleen that drains into the liver, has been a very valuable experimental approach for evaluating both primary tumor growth and metastatic potential [10–12]. However, in most experimental settings, the tumor cells have been implanted into a healthy liver in the animal host, a tissue background hardly resembling those in patients where HCC emerges and progresses, consequently leading to the generation of incomplete or even misleading information on the mechanistic aspects of the pathological process. In this regard, several studies have shown that manipulation of the fibrotic process could result in dramatic effects

on tumorigenesis; however, they have primarily focused on the hepatic stellate cells themselves, with limited probes into the accompanying immune changes in the experimental systems [10, 13–15].

In this study, we addressed the question of whether common fibrotic stimuli could result in alterations to tumor growth in the context of orthotopic implantation models, with accompanying changes in both the immune components and tumor responses to the fibrotic tumor microenvironment. Our findings reveal a markedly different tumor microenvironment and tumor growth kinetics in a clinically relevant fibrotic context, emphasizing the necessity for more accurate modeling that takes into account the drastic changes in the tissue caused by chronic liver disease.

2 Materials and methods

2.1 Mice

All experimental procedures described here were approved by the Duke University Animal Care and Use Committee. C57BL/6 breeding mice were purchased from The Jackson Laboratory.

2.2 Fibrosis induction

For the carbon tetrachloride model of liver fibrosis, 6- to 7-week-old male C57BL/6 mice were treated with either carbon tetrachloride diluted 1:20 in olive oil or olive oil alone at a volume of 600 $\mu\text{L kg}^{-1}$. Mice received biweekly intraperitoneal injections for 6 weeks and were sacrificed 2 days following the 12th injection.

For the bile duct ligation (BDL) model of liver fibrosis, 12-week-old male C57BL/6 mice were anesthetized, and the common bile duct isolated and transected between two ligations. Mice were sacrificed 14 days post-surgery.

2.3 Orthotopic implantation

Implantation occurred either 2 days after the eighth injection of carbon tetrachloride or olive oil, or immediately following BDL. Mice were anesthetized, or maintained under anesthesia in the case of the BDL, and the abdomen opened to expose the liver. 3×10^6 Hepa1-6 cells expressing a GFP-labeled luciferase reporter were suspended in 30 μL of growth factor-reduced Matrigel and injected into the left lobe of the liver. Following implantation, the peritoneum and the skin were closed using surgical suture. Mice were monitored for health and sacrificed 14 days after implantation. In the case of mice with liver fibrosis induced by carbon tetrachloride or their controls, injection of the ninth dose of carbon tetrachloride or olive oil occurred 2 days after surgery, and the 12th dose occurred 12 days after surgery, with sacrifice of the mice occurring 14 days after surgery.

2.4 Sample harvest and preparation

Blood was harvested from the inferior vena cava to examine serum circulating alanine aminotransferase (ALT) and circulating aspartate aminotransferase (AST). Samples were incubated at room temperature for 10 min then centrifuged at 3000 r min^{-1} for 10 min,

following which serum was aspirated and transferred to a new microcentrifuge tube and stored at -80°C until assay.

Livers were harvested and either immediately post-fixed in 10 % phosphate-buffered formalin at 4°C on an orbital shaker for 1 day before being washed with 70 % ethanol and embedded in paraffin blocks, or processed for flow cytometry or fluorescence-activated cell sorting (FACS). Tissue processed for flow cytometry was isolated from the mouse, diced, and ground through a $40\text{-}\mu\text{m}$ -pore filter into DMEM with 10 % FBS and $12\text{ }\mu\text{L}$ DNase I per 2 mLs of DMEM, incubated on an orbital shaker at 37°C for 1 h, resuspended in ACK Lysing Buffer to clear red blood cells, and resuspended in FACS buffer (PBS with 2 % FBS and 2 mmol L^{-1} EDTA). Samples were subsequently blocked with anti-mouse CD16/32, incubated with primary antibodies, washed with FACS buffer, post-fixed with 2 % paraformaldehyde, washed with FACS buffer, and resuspended in FACS buffer and stored at 4°C until evaluation on a BD FACSCanto II (<1 day). Samples processed for FACS and subsequent analyses were treated in a similar way; however, after staining with primary antibodies and washing with FACS buffer, they were resuspended in FACS buffer with $10\text{ }\mu\text{L}$ DNase I per mL and sorted on a BD Diva sorter. Antibodies were purchased from eBioscience (CD4 APC, NK1.1 APC Cy7, CD11b PerCP Cy5.5, Gr-1 Pacific Blue, and F4/80 APC) and Biolegend (LEAF purified anti-mouse CD16/32, TCR β PE Cy7, CD8b FITC, CD25 PE).

2.5 Calculation of the liver as percentage of body weight

At sacrifice, mice were euthanized, weighed, and their livers dissected and weighed. Percent of body weight was calculated as $100\% \times (\text{weight of liver})/(\text{weight of the entire mouse})$.

2.6 Histology

Tissue evaluated by histology was fixed in 10 % phosphate-buffered formalin and embedded in paraffin blocks as described above. Subsequently, $5\text{-}\mu\text{m}$ -thick sections were cut on a microtome, and tissue stained for picrosirius red using standard protocols.

2.7 ALT and AST evaluation

ALT and AST colorimetric quantitative evaluation of circulating ALT and AST was accomplished using kits purchased from Biotron Diagnostics. Levels in the serum of mice with tumor implantation with or without fibrosis induction were determined according to the manufacturer's instructions with appropriate calibration controls.

2.8 qPCR analysis of isolated tumor and stromal cells

At sacrifice, mice were dissected, and samples processed as described before. Samples were suspended in FACS buffer, and sorted based on GFP-positivity; there was a 2 log difference in fluorescence intensity between the sorted GFP $^{+}$ (tumor) and GFP $^{-}$ (stromal) cells. RNA was then extracted from isolated cells, reverse transcribed using iScript, and samples probed for targets of interest.

2.9 Statistics

All statistics were performed using GraphPad Prism. Unpaired *t* tests were performed between each fibrotic treatment and its control, as in, carbon tetrachloride to olive oil, and BDL to sham surgery. Significance was set at the $P < 0.05$ level.

3 Results

3.1 Implantation of cells into a fibrotic liver results in enhanced tumor growth

To directly assess the impact of fibrosis and its concurrent inflammation, we utilized two of the most common models for the induction of fibrosis in the liver: carbon tetrachloride (CCl₄) administration and BDL [6, 9]. CCl₄ administration results in hepatocyte death upon its metabolism to trichloromethyl radicals (CCl₃), resulting in centrilobular necrosis, inflammation, and hepatic stellate cell activation [6, 8, 9]. BDL, on the other hand, leads to cholestatic liver injury and a biliary fibrosis emanating from periportal regions, also with inflammation and hepatic stellate cell activation [6, 9]. The inflammation arising from these models is an intricate and multifaceted process, with the involvement of important common cellular mediators, such as Kupffer cells, infiltrating monocytes, and lymphocytes. In particular, studies have shown important proinflammatory and profibrogenic roles of Gr1⁺ monocytes, antifibrotic roles of NK cells, and profibrotic roles of CD4⁺ and CD8⁺ T cells, through interactions with both other immune cells and the collagen-producing hepatic stellate cells [6, 8, 9]. The cytokines and chemokines produced by parenchymal, nonparenchymal, and immune cells throughout the entire progression of the disease play an important role in determining the course and outcome of the disease. For example, monocytes can be recruited by hepatocyte-originating CCL2 and produce abundant TGFβ, which is important for hepatic stellate cell transdifferentiation, while also producing the inflammatory cytokines TNFα, IL-1β, and IL-6 [9]. More thorough reviews of this complex topic, including analyses of both these models of fibrosis induction, as well as analysis and discussion of important non-cellular mediators of this fibrotic process, can be found in [6, 8, 9]. Consistent with those previous reports, these two models generate robust and reproducible, but slightly different fibrotic phenotypes, as demonstrated by picrosirius red staining for collagen deposition and, as markers for liver damage, significantly elevated levels of the circulating transaminase ALT and moderately elevated AST (Fig. S1). Similar to the symptoms commonly observed in human patients with liver disease, both models induce expansion of the liver mass (measured in this study as a percentage of body weight) due to inflammation and regenerative mechanisms: Livers comprise 6.4 % of the body weight in the CCl₄ model, and 6.6 % of the body weight in the BDL model, compared to 5.0 % in healthy controls. Importantly, orthotopic implantation of Hepa1-6 murine liver tumor cells in C57BL/6 mice under these fibrotic contexts resulted in significantly enhanced tumor growth, even taking into account of the aforementioned expansion of the liver as a percentage of body weight in response to fibrotic stimuli: While the normal liver makes up 5.0 % of the body weight of a mouse, orthotopic implantation in an olive oil-treated mouse led to tumor-bearing livers comprising 5.7 % of the body weight, while CCl₄ administration led to tumor-bearing livers comprising 9.9 % of the body weight, compared to 6.4 % of the body weight without tumor implantation (Fig. 1a, b). Using the BDL model, sham surgeries led to tumor-bearing livers comprising 5.7 % of the body weight, while BDL led to tumor-

bearing livers comprising 10.2 % of the body weight, compared to 6.6 % of the body weight without tumor implantation (Fig. 1c, d). These results demonstrate a critical role for inflammatory fibrotic stimuli in the liver tissue to promote growth of liver tumor cells inoculated orthotopically.

3.2 Inflammatory infiltrate of immune cells into the tumor is altered by fibrotic stimuli

We next examined if this fibrotic context altered the immune components of the tumor microenvironment. After harvesting tumors and making single cell suspensions, we determined the populations comprising the immune cell component of the implanted tumor in mice with or without the induction of fibrosis. As shown in Fig. 2a, b, d, and e, the proportion of natural killer (NK) cells seemed to be slightly reduced in the tumors of mice treated with fibrotic stimuli, and the amounts of T cells, both CD4⁺ and CD8⁺, were similar in the mice treated with fibrotic stimuli compared to their respective controls. Interestingly, there appeared to be some variation between the effects of fibrotic stimuli on CD4⁺ CD25⁺ T regulatory (Treg) cells: BDL enhanced the proportions of these cell types, whereas CCl₄ seemed to leave the amount of these cells unchanged (Fig. 2c–e). On the other hand, tumor-infiltrating CD11b⁺ myeloid cells were strikingly enhanced in the BDL model but not in the CCl₄ model (Fig. 3a, c, d). Importantly, proportions of immune-suppressive cell populations, specifically CD11b⁺ Gr1^{hi} myeloid cells, were drastically increased in tumors implanted into both fibrotic contexts (Fig. 3b–d). Finally, the pattern of F4/80 expression was similar in CD11b⁺ myeloid cells in both models, across all levels of Gr1 expression (Fig. 3e, Fig. S2). It is worth noting that, while there were some commonalities with the immune infiltrate in the fibrotic liver itself (Fig. S3), there were distinctions between the immune infiltrate in the liver tissue and the tumor itself. In the CCl₄ model, there was an enhanced lymphocytic infiltration into the tissue of both NK and T cell subtypes (Fig. S3a). In contrast to the tumor tissue, there was also a significant enhancement in the portion of CD4⁺ T cells which are CD4⁺ CD25⁺ Treg cells (Fig. S3a, b). Interestingly, this was the only alteration in the proportion of lymphocytes recruited: All other cell types evaluated were similarly distributed proportionally between olive oil- and CCl₄-treated mice (Fig. S3b). In the myeloid compartment, there were again differences between the tumor tissue and the non-tumor tissue. While both tumor tissue and non-tumor tissue demonstrated enhanced accumulation of myeloid cells, and particularly in CD11b⁺ Gr1-expressing suppressive cells, those cells expressed only intermediate levels of Gr1 (Gr1^{mid}) in the non-tumor tissue (Fig. S3c, d), in contrast to the high level of Gr1 expression (Gr1^{hi}) in those cells in the tumor tissue (Fig. 3b–d). F4/80 expression was also altered in the infiltrating myeloid cells: Significantly higher expression was observed on both Gr1^{hi} and Gr1^{mid} cells (Fig. S3e), in contrast to the lack of difference in the tumor. Taken together, these results indicate that the immune infiltrate into the tumors is significantly altered by the presence of fibrotic stimuli and that while differing methods of fibrosis induction yield differing microenvironmental compositions, there are important common components, specifically increased immune-suppressive CD11b⁺ Gr1^{hi} myeloid cells, which is distinct from the infiltrate into the surrounding non-tumor tissue.

3.3 Cytokine expression in tumor and stromal cells is altered by a fibrotic microenvironment

Our data so far have demonstrated that fibrosis induction prior to or concurrently with orthotopic tumor implantation alters both tumor growth kinetics and immune infiltration into the tumor. We then explored the effects of this fibrotic microenvironment on both the stromal and tumor cells themselves, particularly on the expression of a panel of immune mediators. Utilizing the CCl₄ model, we labeled the tumor cells with GFP, implanted them into olive oil- and CCl₄-treated mice, and then at harvest sorted the GFP⁻ stromal cells and GFP⁺ tumor cells from the resultant tumors (Fig. S4), extracted their RNA, reverse transcribed, and probed for expression of these mediators. Examining the stromal cells that had infiltrated the tumor, we found that there were indeed differences in cytokine expression by those cells between the two groups (Fig. S5; Table S1). A total of 53 of 93 soluble mediators examined were increased in the CCl₄-treated group compared to the olive oil controls, 45 of which at >1.5 fold, while 40 were decreased, 15 of which at <0.9 fold. The patterns of changes in the expression of those mediators were complex: For example, Th2 cytokines such as IL-5 and IL-13 were reduced in the CCl₄-treated mice, along with IL-2, a cytokine critical for the survival and functionality of activated T cells [16–18]. By contrast, TGFβ and numerous interferons, which have been strongly linked to initiation of and the tissue response to the fibrogenic process [6–9], showed increased abundance. In all, a large population of cytokines and inflammatory mediators were found to display a robustly altered expression, with a mix of functional hits relevant to the immune response to both fibrotic tissue and tumors. When the tumor cells themselves were examined, a different yet complementary pattern to those of the stromal component was observed. Compared to cells grown in culture, tumor cells implanted into both non-fibrotic and fibrotic stimuli increased expression of the majority of mediators probed, and consistently decreased expression of CXCL7 (Fig. 4a; Table S2). The increases or decreases in expression were also relatively consistent between contexts, with only 5 of 61 targets increasing in the fibrotic context while decreasing in the non-fibrotic, and even these targets had very small if not negligible changes in expression (Table S2). More importantly, we found that a large cohort of mediators was increased in the fibrotic context compared to the non-fibrotic (Fig. 4b; Table S3). Indeed, only 11 of 81 targets (6 at <0.9 fold of the olive oil control) examined demonstrated decreased expression in tumor cells in the fibrotic context compared with the non-fibrotic. Notable among these 11 targets were CCL2 and IL-2; CCL2 is a chemotactic cytokine affecting macrophage recruitment, among other functions [19, 20], while IL-2, as mentioned earlier, is critical for the survival and functionality of activated T cells [16–18]. As mentioned, however, the majority of immune mediators (70 of 81; 56 at >1.5 fold of the olive oil control) examined demonstrated increased expression. Of particular note are the dramatic increases in a plethora of chemokines, as well as members of the interferon family and the IL-17 family, especially IL-25 (Fig. 4b; Table S3). IL-17 family members could contribute to the Th17-dominant T helper phenotype in liver cancer, which is linked to poorer prognosis; IL-25 itself has been shown to perform both Th17 and Th2 functions, both of which would lead to worse prognosis for patients [21–26]. These mediators with altered expression could drastically alter both innate and adaptive immune cell function, indicating a systemic reprogramming of immune activity. Together, these data demonstrate that a fibrotic tissue background induces substantial alterations in both the stromal components

and the implanted tumor cells themselves, thus modulating both the number and types of immune cells present as well as their functionality within the tumor microenvironment to promote tumor growth.

4 Discussion and conclusion

Liver cancer is a devastating disease, the progression and prognosis of which are intricately intertwined with the chronically inflamed and diseased tissue in which it develops. This crucial link has been made especially clear by previous studies showing that patterns of gene expression in diseased non-tumor liver tissue of patients with liver cancer is more predictive of survival than tumor tissue [27] and that modulating the fibrosis burden in patients modulates tumor development and progression [14, 28]. However, studies examining the interplay between this altered environment and the tumor, and the effects on the progression of the disease post-malignant transformation, have often been limited or neglected.

In this study, we have demonstrated that fibrosis has a significant effect on tumor growth and progression, corresponding to a marked alteration in the immune composition of the tumor microenvironment, similar to what other groups have previously shown in different systems [10, 13–15]. Importantly, we have identified consistent alterations in the numbers and types of immune cells present in the tumor microenvironment. Specifically, we found that more immunosuppressive cell types, such as neutrophils/myeloid-derived suppressor cells, were accumulated at higher levels in tumors grown in fibrotic livers. These data corroborate previous reports using spontaneous carcinogenesis models, which have demonstrated increased occurrence of these cell types, and patient samples which have correlated the abundance of these cells to worse prognosis of patients [29–32]. These data also help to explain the phenotype of increased tumor burden, and provide opportunities for therapeutic interventions. Myeloid-derived suppressor cells suppress the functionality of T cells as well as NK cells [29–31, 33, 34]. They also promote the differentiation of more immunosuppressive immune cells, thereby greatly hindering anti-tumor immune responses [29]. In the context of liver cancer, Gr1⁺ myeloid cells have recently been shown to modulate tumor burden and the fibrotic microenvironment, similar to our results and stressing the importance of this population of cells in the progression of the disease [32, 35].

In the meantime, there were also differences between the fibrotic models in accumulation of various tumor-infiltrating immune cells. Specifically, BDL resulted in the accumulation of T regulatory cells, while the CCl₄ model resulted in few differences in the T cell populations. These data lead us to conclude that different models may be more useful for studying the contributions of different immune cell populations mechanistically. They also indicate that different etiological factors, which develop different types of fibrosis, may be modeled more robustly using one system over another, as the immune contexture of the tumor is indeed different depending upon the experimental approach by which fibrosis is induced. Human samples as well could more accurately be evaluated in a system that at least partially mimics the effects of the fibrotic environment on the tumor by utilizing the CCl₄ system. Nude mice have been shown to develop fibrosis when treated with this reagent [36, 37], and our data with the use of CCl₄ as the inducer of fibrosis indicate that there are only slight differences in the numbers of tumor-infiltrating T cells. However, data generated using human samples

in this context must be evaluated keeping in mind this incomplete interplay with the microenvironment in the absence of a fully functional immune system. Care must therefore be taken to choose the appropriate model for studying the etiological factor of interest. Most importantly, use of these models will allow for further in-depth study of both transformed cells and tumor-infiltrating immune populations in a more relevant context, and provide opportunities for evaluating experimental manipulations of these cell groups within the microenvironment.

We have also demonstrated that the fibrotic environment results in marked alterations in the transformed cells themselves, with substantial changes in the production of immune mediators, in addition to the altered immune cell populations. First, a dramatic change in the amount of cytokines produced by tumor cells is induced purely by implantation of these cells into an animal, demonstrating a responsiveness of these cells to the new environmental tissue context and requisite adjustments. Second, and more importantly, the cytokines produced by the tumor cells were dramatically altered when a fibrotic stimulus was added to the system. These alterations encompassed cytokines crucial for recruitment and functionality of both innate and adaptive immune cells, and were indicative of a systemic shift in production of mediators by tumor cells. Thus, in order to study the tumor cells themselves and their interactions with their inflammatory environment, relevant models should be utilized; without this context, potent mediators of tumorigenesis and progression may not be detected, or have altered functionality that may or may not be relevant to the pathological process associated with HCC.

Many models have so far been generated to study HCC tumorigenesis and metastasis utilizing diverse methodologies, such as implantation of tumor cells in various locations, administration of chemical carcinogens, or transgenic manipulations. Our data indicate that caution must be taken to evaluate data from these models in regard to the context of the non-tumor tissue and the tumor microenvironment. Fibrotic stimuli, either chemical or mechanical, can be applied concurrently with transplantation to modulate the tumor development and the context in which it develops, adding a more relevant inflammatory context to experiments [10, 12, 13]. This context exerts a powerful impact on tumor growth, and indeed also affects tumor progression: Addition of CCl₄ has been shown to be necessary for tumor development following inoculation of HCC progenitors intrasplenically [12], and even to significantly increase the number of metastatic lung lesions after orthotopic implantation of transformed cells into the fibrotic liver [10], demonstrating a powerful role for fibrosis and inflammation in modulating the progression and metastasis of transplanted tumors in those models. The classic chemical carcinogenesis model involves administration of diethylnitrosamine (DEN), a hepatocyte DNA adduct-inducing agent that results in consistent tumor establishment within a year [20, 38, 39]. Inflammatory mechanisms are found to regulate tumor development in the DEN model [20, 39]; importantly, the model can also be combined with fibrotic stimuli, resulting in quicker tumorigenesis as well as more robust inflammatory processes [12, 15, 40]. A wide variety of transgenic models are also prevalent; many utilize classic oncogenes or tumor suppressors such as myc or p53, or liver-specific expression of hepatitis viral proteins such as HBx, HBsAg, or HCV core and envelope proteins [41, 42]. These models have proven their worth in providing valuable insights into the functionalities of different signaling pathways and their effects on the

transformed cells. They also tend to have an inflammatory component as well, making them more relevant to the clinical situation. However, they generally do not develop fibrosis, which, as shown here, has large effects on both the tumor growth and its immunological profile. Other transgenic models model the entire inflammatory tumorigenic process, such as TGF β receptor-deficient mice, MDR2^{-/-} mice, or liver-specific lymphotoxin-expressing mice [14, 43–46]. When used appropriately, these models develop liver fibrosis, inflammation, and HCC, thus closely modeling the clinical development of the disease [14, 43–46].

Taken together, our work provides evidence that fibrotic stimuli further enhance tumor growth kinetics, even in the established tumor. We have also shown that the immune microenvironment of these tumors is drastically skewed by fibrotic stimuli and that these stimuli result in the accumulation of different immune cell types. Importantly, there are both commonalities and dissimilarities between these models, indicating that perhaps there are both common and specific immune mechanisms across multiple types of fibrosis, and based on the etiological factor chosen for study, some models may be more suitable than others. In all, use of relevant inflammatory models in liver cancer studies is necessary for accurately answering key questions and generating insights, both mechanistically and therapeutically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Orthotopic implantation of tumor cells into a fibrotic liver results in enhanced tumor growth. Implantation of 3×10^6 Hepa1-6 cells suspended in 30 μ L of growth factor-reduced Matrigel into the livers of mice either treated with carbon tetrachloride (**a, b**) or subjected to bile duct ligation (**c, d**) resulted in significant enhanced tumor growth compared with either olive oil administration (**a, b**) or sham surgery (**c, d**) controls. ** $P < 0.01$, **** $P < 0.0001$

Fig. 2.

Lymphocytic immune contexture of tumors developed in a fibrotic liver is different from those developed in a healthy liver. (a) Representative flow cytometry contour dot plots of tumor-infiltrating NK1.1⁺ and TCRβ⁺ cells in C57Bl/6 mice treated with (from left to right) olive oil, carbon tetrachloride, sham surgery, or bile duct ligation and tumor implantation. (b) Representative flow cytometry contour dot plots of TCRβ⁺ tumor-infiltrating CD8⁺ and CD4⁺ cells in C57Bl/6 mice treated with (from left to right) olive oil, carbon tetrachloride, sham surgery, or bile duct ligation and tumor implantation. (c) Representative flow

cytometry contour dot plots of TCR β ⁺ CD4⁺ tumor-infiltrating CD25⁺ cells in C57Bl/6 mice treated with (from left to right) olive oil, carbon tetrachloride, sham surgery, or bile duct ligation and tumor implantation. **(d)** Quantification of NK1.1⁺, NK1.1⁺TCR β ⁺, TCR β ⁺, TCR β ⁺CD4⁺, TCR β ⁺CD8⁺, and TCR β ⁺CD4⁺CD25⁺ cells in tumor tissue from mice in **(a)**, **(b)**, and **(c)** as percentage of gate. **(e)** Quantification of Singlets (live single cells based on FSC-A and FSC-H analyses following SSC-A and FSC-A gating), NK1.1⁺, NK1.1⁺TCR β ⁺, TCR β ⁺, TCR β ⁺CD4⁺, TCR β ⁺CD8⁺, and TCR β ⁺CD4⁺CD25⁺ cells in tumor tissue from mice in **(a)**, **(b)**, and **(c)** as percentage of total recorded events. * $P < 0.05$, *** $P < 0.001$

Fig. 3.

Myeloid immune contexture of tumors developed in a fibrotic liver is different from those developed in a healthy liver. **(a)** Representative flow cytometry contour dot plots of tumor-infiltrating CD11b⁺ and TCRβ⁺ cells in C57Bl/6 mice treated with (from left to right) olive oil, carbon tetrachloride, sham surgery, or bile duct ligation and tumor implantation. **(b)** Representative flow cytometry contour dot plots of Gr1 expression on CD11b⁺ tumor-infiltrating cells in C57Bl/6 mice treated with (from left to right) olive oil, carbon tetrachloride, sham surgery, or bile duct ligation and tumor implantation. **(c)** Quantification

of CD11b⁺, CD11b⁺Gr1^{hi}, CD11b⁺Gr1^{mid}, and CD11b⁺Gr1^{lo} cells in tumor tissue from mice in (a) and (b) as percentage of gate. (d) Quantification of Singlets (live single cells based on FSC-A and FSC-H analyses following SSC-A and FSC-A gating), CD11b⁺, CD11b⁺Gr1^{hi}, CD11b⁺Gr1^{mid}, and CD11b⁺Gr1^{lo} cells in tumor tissue from mice in (a) and (b) as percentage of total recorded events. (e) Quantification of mean F4/80 fluorescence intensity of CD11b⁺ cells in tumor tissue from mice in (a). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

Fig. 4. Soluble immune mediator expression by tumors developed in a fibrotic liver is markedly different from that in a healthy liver. Tumors were harvested at 14 days post-implantation, and a single cell suspension was made. Tumor cells expressing a GFP-luciferase vector were sorted from the suspension, their RNA extracted and reverse transcribed, and expression of soluble immune mediators examined. **(a)** Comparison of expression of cytokines in Hepa1-6 GFP-luciferase cells isolated from tumors implanted into either olive oil (red)- or carbon tetrachloride (blue)-treated mice, normalized to expression of the same cytokines in the same cell line maintained in tissue culture. **(b)** Comparison of expression of cytokines in Hepa1-6 GFP-luciferase cells isolated from tumors implanted into either olive oil (red)- or carbon tetrachloride (blue)-treated mice, normalized to average expression of these factors in tumor cells implanted into mice that were treated with olive oil. *Sci. Bull.*