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Successful Chemoimmunotherapy against Hepatocellular Cancer in a Novel Murine Mode

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Abstract

Background and Aims—We have established a clinically relevant animal model of hepatocellular cancer (HCC) in immune competent mice to elucidate the complex dialog between host immunity and tumors during HCC initiation and progression. Mechanistic findings have been leveraged to develop a clinically feasible antitumor chemoimmunotherapeutic strategy.

Methods—Intraperitoneal injection of carbon tetrachloride and intrasplenic inoculation of oncogenic hepatocytes were combined to induce progressive HCCs in fibrotic livers of immunocompetent mice. Immunization and adoptive cell transfer (ACT) were used to dissect the tumor antigen-specific immune response. The ability of the tyrosine kinase inhibitor sunitinib to enhance immunotherapy in the setting of HCC was evaluated.

Results—This new mouse model mimics human HCC and reflects its typical features. Tumor-antigen-specific CD8⁺ T cells maintained a naïve phenotype and remained responsive during early-stage tumor progression. Late tumor progression produced circulating tumor cells, tumor migration into draining lymph nodes, and profound exhaustion of tumor-antigen-specific CD8⁺ T cells associated with accumulation of PD-1^{hi} CD8⁺ T cells and regulatory T cells (Tregs). Sunitinib-mediated tumoricidal effect and Treg suppression synergized with antibody-mediated blockade of PD-1 to powerfully suppress tumor growth and activate anti-tumor immunity.

Conclusion—Treg accumulation and upregulation of PD-1 provide two independent mechanisms to induce profound immune tolerance in HCC. Chemoimmunotherapy using FDA-approved sunitinib with anti-PD-1 antibodies achieved significant tumor control, supporting translation of this approach for the treatment of HCC patients.

Graphical abstract

Chemoimmunotherapy against HCC

Keywords

Hepatocellular cancer (HCC); Chemoimmunotherapy; Regulatory T cells (Tregs); Programmed cell death protein 1 (PD-1); Sunitinib; Cancer; Circulating tumor cell (CTC); Immune checkpoint

INTRODUCTION

Hepatocellular cancer (HCC) remains one of the deadliest cancers with limited clinical options[1]. HCC is refractory to classic chemotherapy and unsuitable for radiation treatment due to liver toxicity[2]. Surgical resection or ablation offers a small chance for cure. Liver transplantation is an effective treatment for cirrhosis and early tumors, but most patients are ineligible because recurrence is common and organs are scarce[3]. The receptor tyrosine kinase inhibitor (RTKI), sorafenib, was the first and only drug approved by the Food and Drug Administration (FDA) to treat unresectable HCC in 2008; however, sorafenib only increases the median overall survival of patients from 7.9 to 10.7 months[4]. This small but statistically-significant therapeutic effect highlights the challenge in treating this devastating disease.

Immunotherapy has been successfully used in the treatment of several human cancers, including melanoma[5] and lung cancer[6], where high objective response rates have been obtained. Recent trials with immunotherapy also have shown promise against HCC[7], but no approaches have been translated into clinical application[8, 9]. In particular, overcoming tumor-induced immune tolerance in HCC is still an extremely challenging task[10, 11]. Lack of a clinically relevant model is a critical barrier to understanding HCC immunity and developing immunotherapeutic strategies.

Due to the aggressive nature of HCC and its tendency to arise in the setting of inflammation and liver fibrosis, establishing a clinically faithful model has been challenging[12]. We previously used intrasplenic (ISPL) inoculation of histologically normal hepatocytes from MTD2 mice into immunocompetent wild type mice to generate an orthotopic HCC model, but this model lacked the element of liver fibrosis/cirrhosis which is present in the vast majority of patients with HCC[13, 14]. In the present study, IP injection of CCl₄ to induce

liver fibrosis is integrated into this strategy. As a result, tumors arise in the context of liver fibrosis in immunocompetent mice, mimicking the clinical scenario of human HCC.

The cytotoxic T-lymphocyte response has been identified as a powerful and effective link in the anti-tumor immune network[15, 16]; however, more than 96% of tumor-infiltrating lymphocytes (TILs) in HCC have been demonstrated to be quiescent[17], losing their cytotoxic function toward tumor cells. Also, tumor-induced increase in Tregs is observed in HCC patients and is associated with a poor prognosis[18, 19]. In addition, upregulation of inhibitory immune checkpoints such as CTLA-4 and PD-1 has been recognized to mediate peripheral T-cell tolerance and silence the immune response[20-22]. These data highlight the relationship between immune inactivation and tumor progression[23]. However, identification of key factors mediating tumor-induced profound immunotolerance for the development of immunotherapy against HCC remain to be determined.

In the present study, we establish and characterize a clinically relevant murine model and use this model to determine the interplay of host immunity and tumors in the progression of HCC. Critically important factors identified in tumor-induced immunotolerance are targeted to develop a chemoimmunotherapeutic-based approach for HCC control.

MATERIALS AND METHODS

Mice

Line MTD2 [24] and 416[25] mice served as the source of tumorigenic hepatocytes and tumor antigen-specific (TAS) CD8⁺ T cells (TCR-I T cells), respectively. Male C57BL/6 mice from Jackson Laboratory (Bar Harbor, ME) were used as recipient mice in our HCC model. Animal experiments were approved by the IACUCs of the Medical University of South Carolina, Pennsylvania State University College of Medicine, and University of Missouri. All mice received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals”.

IP administration of CCl₄, ISPL injection of hepatocytes and magnetic resonance imaging (MRI)

10% CCl₄ (v/v) solution in corn oil was IP injected into C57BL/6 mice twice a week at 8 mL/kg of body weight (BW) for three and six weeks[26, 27]. Two weeks after the last injection, mice receive TAg-transgenic hepatocytes isolated from young male MTD2 mice by ISPL injection[13]. MRI scans for tumor surveillance were obtained on a 7.0 Tesla system (Bruker Biospin, Billerica, MA, USA) with in-plan resolution of 0.1 mm and 1 mm slice thickness. A detailed description of the resultant model is provided in Supplementary Materials and Methods.

Flow cytometric analysis

Ex vivo staining of lymphocytes from spleen and tumors with MHC tetramers and fluorochrome-labeled antibodies was performed on single-cell suspensions as described[13]. Stained cells were analyzed with a FACScan flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). Staining for intracellular IFN- γ and TNF- α was

performed as described previously[13]. Staining for FoxP3 was performed with the staining buffer set from eBioscience using the manufacturer's recommendations.

***In vivo* TCR-I T-cell proliferation assay**

RBC-depleted TCR-I T cells derived from spleens and lymph nodes (LNs) of line 416 mice were labeled with 5 μ M carboxy fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) as previously described [25]. The CFSE-labeled TCR-I T cells were intravenously (IV) injected into mice at a dose of 1×10^6 cells/mouse in 0.2 ml volume. Seven days after adoptive transfer the dilution of CFSE in TCR-I T cells was determined by flow cytometry. Detailed information regarding TCR-I transgenic T cells and their adoptive transfer is provided in Supplementary Materials and Methods.

Capturing and Immunostaining of CTCs

Early-stage and late-stage tumor-bearing mice were anaesthetized by inhalational isoflurane to harvest blood by cardiac puncture. Microfilters developed by CreatV are used to capture CTCs by size-based exclusion according to the manufacture's protocol[28] (Detailed information in Supplementary Materials and Methods). Filters containing cells were fixed with 4.0% formaldehyde for 15 min, washed three times with $1 \times$ phosphate-buffered saline (PBS), permeabilized with 0.3% Triton X-100, and blocked in 1% bovine serum albumin (BSA) in $1 \times$ PBS for 1h at room temperature (RT). Primary antibodies for EpCam, TAg, or cytokeratin were incubated overnight at 4°C at a 1:100 dilution in $1 \times$ PBS containing 1% BSA. Overnight incubation was followed by 3×10 mins washes with $1 \times$ PBS, followed by incubation in appropriate Dylight488 secondary Ab for 2h in the dark at RT. DAPI was used for nuclear counterstaining. Filters were examined under an immunofluorescence microscope (Nikon ECLIPSE 90i), and images captured using NIS-Elements AR3.2 software.

Histologic staining and Immunohistochemistry (IHC)

Liver biopsies were fixed with 10% neutral buffered formalin and embedded in paraffin. Tissue sections were processed and stained with hematoxylin–eosin (H&E), Masson's trichrome and picrosirius red as described[29]. IHC to detect α -SMA was performed as described[30].

Sunitinib and anti-PD-1 administration, adoptive cell transfer, and immunization

Sunitinib was orally administrated at 20 mg/kg in 0.2 mL of vehicle buffer every other day for two weeks. Anti-PD-1 Abs were IP injected into each mouse at 0.2 mg in 0.15 mL twice a week for 4 weeks. For ACT, 1×10^6 TCR-I T cells isolated from spleens and lymph nodes of line 416 mice were suspended in 0.2 mL of HBSS and injected IV into the tail vein. For immunization, 3×10^7 freshly harvested B6/WT-19 cells were suspended in 0.2mL of PBS and IP injected into each mouse. Detailed information including sunitinib and anti-PD-1 administration, ACT, and IP immunization of B6/WT-19 cells is provided in Supplementary Materials and Methods.

Western-blotting, lymphocyte isolation, PCR and primers, peptides, reagents, antibodies, cell line, dissection of liver draining LNs, and MRI based quantification of tumor volume are described in Supplementary Materials and Methods.

Statistics

Paired data were analyzed using a 2-tailed paired Student's *t* test. A *P* value of less than 0.05 was considered significant.

RESULTS

Establishment of a clinically relevant murine model with typical features of human HCC

To induce liver fibrosis, CCl₄[26] was administered to male C57BL/6 mice twice a week for 3 or 6 weeks (**Fig 1a**). Two weeks after the last injection, treated mice received ISPL injection of histologically normal hepatocytes isolated from young male MTD2 mice that express TAg (**Fig 1a**)[13]. Macroscopic examination revealed hepatic tumor masses began to appear about one month after inoculation and grew progressively (**Fig 1b**). Extensive histological evaluation (**Fig 1c**) indicated that liver tissues in recipient mice exposed to vehicle show normal architecture; 3 or 6 weeks of CCl₄-treatment led to a markedly distorted hepatic architectural pattern, including bridging portal fibrous septa, notable portal ductal proliferation with infiltration of multiple random discrete mixed inflammatory cells consisting of variable proportions of macrophages, neutrophils, lymphocytes, and plasma cells. Both Picrosirius-red and Masson's trichrome staining demonstrated that CCl₄ treatment induced increased expression of collagen. This process formed networks resulting in bridging hepatic fibrosis in both the portal structures and centrilobular areas, while control oil treatment was indistinguishable from untreated controls and did not produce any pathological changes. Expression of α -SMA, a marker of activated stellate cells and myofibroblasts, was strikingly increased in the livers from CCl₄-treated mice. Two pathologists independently performed histologic analysis. All of the microscopic features observed in the experimentally-induced murine HCCs are also described in human HCC. Indeed, morphological characteristics of the lesions in the murine and human HCCs are very similar on both macroscopic and pathologic examination. (**Supplementary figure 1**). Quantification of tumorigenic foci by MRI showed a positive correlation with CCl₄ injection with longer CCl₄ treatment associated with more tumor foci (**Fig 1d and Supplementary figure 2**). Macroscopic and IHC evaluation of spleens, lungs and kidneys did not reveal the presence of tumors (unpublished observations), indicating that tumor initiation and progression are liver-specific. As is consistent with human HCC, a notable increase of AFP and GPC3 was detected by immunoblot in the liver tissue of tumor-bearing mice compared to that in tumor-free mice (**Fig 1e**). Both antigens serve as markers for surveillance, diagnosis[31], and potential targets for immunotherapy[32].

These results establish a model in which tumors with increased expression of embryologic HCC-associated antigens arise in the setting of liver fibrosis in immunocompetent mice. This model recapitulates key characteristics of human HCC.

Shift of antitumor immunity from ignorance to tolerance with progression from early stage to advanced stage tumors

To evaluate the host immune response during tumor initiation and progression, cell-based immunization (**supplementary figure 3**) was utilized to determine the expansion and activity of TAS CD8⁺ T cells in tumor-bearing mice at both early and late stages of tumor progression (**Fig 2a**). Immunization at the early-stage with TAg-transformed B6/WT-19 cells strongly stimulated the accumulation of TAS CD8⁺ T cells recognizing TAg epitope I and epitope IV at levels similar to that obtained in tumor-free mice (**Figs 2b-2c**). T cell accumulation was accompanied by increased production of IFN- γ and TNF- α following a short *in vitro* stimulation with peptides corresponding to TAg epitope-I or -IV (**Fig 2d-2e**). Conversely, immunization failed to stimulate accumulation of TAS CD8⁺ T cells in late-stage tumor-bearing mice as evidenced by lack of MHC tetramer⁺ or cytokine-producing cells (**Fig 2b-2e**). Representative dot plots for CD8⁺ T-cell accumulation and production of IFN- γ and TNF- α are shown in **Supplementary Figure 4**.

To further determine the role of tumor microenvironment in inducing immune suppression, TCR-I T cells, expressing a clonal TCR specific for TAg epitope I[25], were isolated from line 416 mice and labeled with CFSE, then adoptively transferred into tumor-free or tumor-bearing mice at either early or late stage. No significant CFSE-diluted TCR-I T cells were detected in either tumor-free or early-stage tumor-bearing mice without immunization, suggesting lack of antigen recognition (**Figs 3a-3b**); however, immunization with B6/WT-19 cells significantly triggered proliferation of TCR-I T cells in both settings. In contrast, a significant proportion of TCR-I T cells had diluted CFSE in late-stage tumor-bearing mice in the absence of immunization, but immunization did not further expand TCR-I T cells (**Figs 3a-3b**). In addition, a short *ex vivo* stimulation with TAg epitope-I peptide did not induce IFN- γ production by TCR-I T cells from immunized late-stage tumor-bearing mice (**Fig 3c-3d**), while notable IFN- γ production was detected in TCR-I T cells from immunized tumor-free mice and early-stage tumor-bearing mice (**Fig 3c-3d**). Representative results for Fig 3c-3d are shown in **Supplementary Fig 5**.

These results suggest that late stage tumor development induces a highly suppressive microenvironment which suppresses activation of both endogenous and exogenous TAS CD8⁺ T-cells in response to TAS immunization. Conversely, tumor-bearing mice at an early stage maintain a naïve environment similar to tumor-free mice and maintain response to immunization.

Migration of tumor cells to draining lymph nodes and their circulation in the blood are associated with immune tolerance in late-stage tumor-bearing mice

A lack of tumor cell migration to draining lymph nodes (DLNs) could explain TAS immune ignorance during early tumor development. To test this hypothesis, DLNs, non-DLNs, and liver tissue were harvested from early and late stage tumor-bearing mice for isolation of genomic DNA. The isolated genomic DNA was probed to detect TAg by PCR[33]. The sensitivity of the assay was determined *in vitro* by mixing the indicated numbers of TAg-positive lymphocytes from transgenic MTD2 mice with a constant number of lymphocytes (1×10^6) from C57BL/6 mice. As few as five TAg-positive cells could be detected by PCR

in the mixed cell population (**Fig 4a**). In the liver tissue from either early- or late-stage tumor-bearing mice, a prominent PCR signal could be detected (**Fig 4b**). In contrast, this specific signal was only detected in the DLNs of tumor-bearing mice at late-stage. No band was detected in DLNs and non-DLNs of tumor-bearing mice at early stage (**Fig 4b**). Thus, localization of tumor cells to the draining LNs may be associated with immunological tolerance while their absence from LNs in mice with early-stage tumors is associated with immune ignorance.

Detection of tumor-specific TAg in the DLNs prompts us to investigate whether CTCs appear in the blood of tumor-bearing mice. With a new and simple microfilter developed by CREATV MicroTech, CTCs were isolated from a small amount of blood by size exclusion. Captured cells from late-stage tumor-bearing mice stained positive for EpCam (epithelial cell adhesion molecule; **Fig 4c**) and Cytokeratin (epithelial differentiation marker; **Fig 4c**), but were negative for CD11b (macrophage marker; **Fig 4d**). Both EpCam and Cytokeratin are commonly used as CTC markers. Tumor-specific TAg was also detected in these cells. Conversely, these cells were not detected in the blood of early-stage tumor-bearing mice. These results indicate that tumor cells shed from tumors and access the blood in late tumor development.

Upregulation of inhibitory immune checkpoints and increase of Tregs are associated with exhaustion of effector CD8⁺ T cells in late-stage tumor development

To assess tumor-induced exhaustion of TILs, the expression of PD-1 and CTLA-4 in immune cells was detected by flow cytometry. Compared to normal mice, the expression of CTLA-4 and PD-1 on CD4⁺ T cells was increased in the splenocytes and TILs in late-stage tumor-bearing mice (**Fig 5a-5d**). Also remarkable was the 29-fold increase in PD-1⁺CD8⁺ T cells detected in TILs (**Fig 5d**). These increases were not observed in early stage tumor-bearing mice (unpublished observations). Thus, enhanced expression of inhibitory immune checkpoints was associated with exhaustion of CD8⁺ T cells in late stage tumor development.

Accumulation of suppressive Tregs[34] and MDSCs[35], associated with tumor-induced immune tolerance[36], was determined by flow cytometry as CD4⁺CD25⁺ FoxP3⁺ Tregs and CD11b⁺Gr-1⁺ MDSCs. No significant difference was detected in the frequency of Tregs and MDSCs between early-stage tumor-bearing mice and normal C57BL/6 mice (**Fig 5e**), but both cell populations were significantly increased in splenocytes and TILs of late stage tumor-bearing mice (**Fig 5e and 5f**). In particular, Tregs increased 2-fold among TILs (**Fig 5f**). Representative plots for Fig 5e-5f are shown in **Supplementary Fig 6**. This increase was not solely induced by CCl₄ injection (**Supplementary Fig 7**). Taken together, these data suggest that increased regulatory cell populations along with upregulation of PD-1 may promote tumor-induced immunotolerance in late-stage tumor-bearing mice.

Combination of sunitinib and anti-PD1 antibody strikingly suppresses tumor growth and powerfully activates TAS anti-tumor immunity

Since increased Tregs and expression of PD-1 in TILs are associated with tumor-specific tolerance, inhibition of Tregs and PD-1 signaling may strongly activate anti-tumor immunity

to suppress growth of established tumors. Size-matched tumor-bearing mice were randomly assigned to receive the following treatments: vehicle control, sunitinib, anti-PD-1 Ab, or a combination of sunitinib and anti-PD-1 Ab (**Fig. 6a**). By week 2, the size of tumors in the vehicle control group were about 2000 mm³. These animals were euthanized as per the IACUC policy. However, tumor growth was significantly delayed in the three treatment groups (**Fig. 6b**). By week 4, over a 25-fold increase in tumor size was detected in the sunitinib-treated group, and over a 15-fold increase was observed in the anti-PD-1-treated group. In contrast, less than an 11-fold increase was detected in mice that received combined therapy (**Fig. 6b-6d**). This statistically significant difference in efficacy clearly indicates that this chemoimmunotherapeutic approach is superior to monotherapy with either sunitinib or anti-PD-1 antibody.

To investigate whether the extent of tumor suppression was positively associated with activation of anti-tumor immunity, the impact of each treatment on the anti-tumor immune response in tumor-bearing mice was investigated. In vivo treatment of tumor-bearing mice with sunitinib or anti-PD-1 Ab alone resulted in an obvious reduction in the frequency of Tregs and CD8⁺ TILs expressing PD-1 (**Fig. 6e-6f**). Reduced PD-1 expression was not due to competitive binding between the therapeutic anti-PD1 mAb and the labeled detection anti-PD-1 mAb (**Supplementary Fig 8**). Importantly, the combination of sunitinib and anti-PD-1 Ab generated a further decrease in the frequency of Tregs and CD8⁺ TILs expressing PD-1 (**Fig. 6e-6f**). Combined therapy also yielded an increase in TAg epitope-I and -IV peptide-induced IFN- γ production by CD8⁺ T cells compared to either monotherapy (**Fig. 6g**). These results demonstrate that combined chemoimmunotherapy optimally promotes the anti-tumor immune response to suppress the growth of HCC.

DISCUSSION

In this study, we developed an innovative and clinically relevant murine model of HCC which recapitulates characteristics of human disease. This in vivo system is both accessible and reproducible and relies on normal immune competent C57BL/6 mice. Over the course of tumor progression TAS immunity is shifted from ignorance to tolerance in association with the appearance of CTCs and an increased frequency of Tregs and PD-1-expressing TILs. Sunitinib in combination with anti-PD-1 Abs generates a synergistic effect, resulting in increased anti-tumor immunity and remarkable suppression of tumor growth.

The establishment of a clinically relevant murine model with the typical features of human HCC[37] is an important contribution of the present study. In this model, initial administration of CCl₄ induces liver injury and fibrosis. Following orthotopic inoculation, the oncogenic hepatocytes are triggered by androgen in recipient male mice to initiate TAg expression under control of a liver-specific promoter. TAg binds and inactivates tumor suppressor factors p53 and Rb[38, 39]. As a result, these transferred hepatocytes become cancer cells and form a tumor mass in the setting of liver fibrosis/cirrhosis. Late tumor development is associated with immunotolerance, tumor cell migration to DLNs, CTCs in the bloodstream, an increase of Tregs and MDSCs, and dysregulation of immune checkpoints, which mimick the clinical scenario of human HCC. As widely recognized human HCC associated antigens, the prominent expression of AFP and GPC3 in this model

provides clinically relevant targets to advance immunotherapeutic translational studies[40-43]. We also optimized MRI parameters to monitor tumor growth in mice from a very early stage. Thus, the new model coupled with established tools provide a unique platform for the investigation of HCC pathogenesis and pre-clinical evaluation of various anti-cancer therapeutic strategies.

In the current study, Tregs and PD-1 were identified as critical factors to mediate HCC-induced immunotolerance. We observed immune ignorance in tumor-bearing mice at an early stage, but immune tolerance at the later stage of tumor growth [33, 35, 44]. In early stage tumor-bearing mice, endogenous and exogenous adoptively transferred TAS CD8⁺ T cells maintained a naïve phenotype. Immunization with tumor-specific antigens was able to activate these cells, facilitating their proliferation and cytokine production. With late-stage tumor development, concomitant with tumor migration into the DLNs and the occurrence of CTCs in the blood, the immunosuppressive microenvironment was induced to inactivate endogenous TAS CD8⁺ T cells and quickly tolerize adoptively transferred TAS CD8⁺ T cells (TCR-I T cells), leading to unresponsiveness to immunization. Similar to human HCC, this immunotolerance is associated with increased frequency of Tregs and expression of PD-1 by TILs[45]. Therefore, sunitinib-mediated blockade of Tregs in combination with Ab-mediated blockade of PD-1 generated a synergistic effect in activating anti-tumor immunity, evidenced by a dramatic increase in the production of IFN- γ by TAS CD8⁺ T cells and a particularly remarkable reduction in PD-1 expression by CD8⁺TILs (**Fig.6**). These non-redundant effects further demonstrate the unique function of sunitinib and anti-PD-1 Ab on tumor-induced immune tolerance in HCC. Collectively, our data suggest that sunitinib and anti-PD-1 function through independent mechanisms to prevent HCC-induced profound immune tolerance.

Development of a powerful and clinically feasible chemoimmunotherapeutic strategy against HCC is another important contribution. In the present study, the combination of sunitinib and anti-PD-1 is apparently superior to either monotherapy and achieves a favorable effect on tumor control. Both human and animal studies from our group and others suggested that the FDA-approved receptor TKI sunitinib exerts direct tumoricidal effects but also activates anti-tumor immunity involved in the suppression of Tregs[13, 46]. Multiple studies demonstrate that immune checkpoint molecules have an essential role in immune evasion of tumor cells [47]. Ab-mediated blockade of PD-1 has generated impressive clinical results and was recently approved by the US FDA for the treatment of human advanced melanoma, bladder, and lung cancer [6, 11, 48]. Specifically for HCC, the interim analysis of the phase I/II nivolumab (humanized anti-PD-1 monoclonal antibody) trial in patients shows a high response rate with long-lasting durable responses [49]. Thus, this combined therapeutic strategy has high translational potential and can be quickly translated for human HCC treatment. We are initiating this phase I clinical trial in patients with HCC.

Sorafenib and sunitinib represent 2 novel small molecule compounds that block multiple receptor tyrosine kinases. Both agents demonstrate an improved progression-free survival and overall survival in patients with different cancers and led to FDA-approval of sorafenib in metastatic renal cell carcinoma (RCC) and HCC, sunitinib in gastrointestinal stromal tumor (GIST), RCC, and pancreatic neuroendocrine tumors (pNET). However, sunitinib,

rather than sorafenib, was chosen for this innovative combinational treatment as we (**supplementary figure 9**) and others have demonstrated that sunitinib potently activates the anti-tumor immune response, while sorafenib has been shown to suppress immune activation [50]. In addition, considering the intolerance of HCC patients toward sunitinib, we reduced the dosage of sunitinib to half of the clinically equivalent dose and combined it with anti-PD-1 Ab in the present study. This combinational approach, achieved more efficient tumor control in comparison with either monotherapy. These results further demonstrate the value of sunitinib in chemoimmunotherapy for HCC treatment.

Apart from the contributions mentioned above, the current study also demonstrates the occurrence of CTCs in the bloodstream in late-stage tumor-bearing mice. Consistent with human disease, these cells do not commonly produce gross metastasis. We used a microfilter with a pore size of about 7 μ M to capture rare CTCs[51]. The captured cells positively stained with Epcam and cytokeratin as well as specific TAg [52], but not CD11b. This suggests that these cells originated from primary tumors and does not represent the fusion of tumor cells with macrophages and monocytes. Lack of tumor cell and macrophage/monocyte fusion may explain why HCC is observed to have a low metastatic potential.[53].

In summary, this unique model provides an opportunity to better understand HCC pathogenesis and a framework to design and assess distinct therapeutic strategies for the treatment of HCC. Increase of Tregs and upregulation of PD-1 function as two independent mechanisms to induce immune tolerance. Combined chemoimmunotherapy with FDA-approved sunitinib and anti-PD-1 Ab achieves optimal tumor control and has a high potential to be rapidly introduced into clinical trials for patients with HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

AFP	α -fetoprotein
CCl₄	Carbon tetrachloride
CFSE	Carboxy fluorescein diacetate succinimidyl ester
CTC	Circulating tumor cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4

GPC3	Glypican 3
H & E	Hematoxylin and eosin
HCC	Hepatocellular cancer
IF	Immunofluorescence
IHC	Immunohistochemistry
IP	Intraperitoneal
ISPL	Intra-splenic
IV	Intravenous
MDSC	Myeloid-derived suppressor cell
MRI	Magnetic resonance imaging
PD-1	Programmed cell death protein 1
RT	Room temperature
TAA	Tumor-associated antigen
TAg	SV40 T antigen
TAS	Tumor antigen-specific
TCR	T cell receptor
TIL	Tumor-infiltrating lymphocyte
Treg	Regulatory T cell
TSA	Tumor-specific antigen

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A lay Summary

In the current study, we have established a clinically relevant mouse model which mimics human liver cancer. Using this unique model, we studied the response of the immune system to this aggressive cancer. Findings from this trial have led to the development of an innovative and clinically feasible chemoimmunotherapeutic strategy.

Figure 1. A novel murine model with typical features of human HCC

(a) The experimental design for establishing an innovative murine model of HCC. C57BL/6 mice were first treated with IP injection of CCl₄ for three or four weeks to induce liver fibrosis. After 2 weeks, the CCl₄-treated mice received normal-appearing hepatocytes isolated from young male MTD2 mice via ISPL inoculation. (b) Representative images of liver tumors in recipient mice with or without CCl₄ treatment. (c) Representative images of liver sections with the indicated staining. Upper panel: hematoxylin and eosin (H&E) staining, asterisks depict forming nodules, arrowheads point to bile ductal proliferation. Second panel: picrosirius red staining; arrows point to abnormal collagen deposition. Third panel: Masson's trichrome staining. Fourth panel: α-SMA immunohistochemistry. Bottom panel: H&E staining, yellow arrows point to neutrophils, green arrows to macrophages, black arrows to lymphocytes, and the blue arrow to a plasma cell. (d) The number of tumorogenic foci in three cohorts is counted based on the MRI image seven weeks after treatment with CCl₄. The cumulative results are presented; * $p<0.05$, ** $p<0.01$; $n=8$; error bars represented mean $\pm$ SD. (e) A representative immunoblot demonstrates increased expression of AFP and GPC3 in tumor-bearing mice compared to normal mice.

Figure 2. Immune response of endogenous TAS CD8⁺ T cells in early-stage and late-stage tumor-bearing mice

Tumor-free mice and tumor-bearing mice with tumor volume $<100 \text{ mm}^3$ and $>400 \text{ mm}^3$ received IP immunization with 3×10^7 B6/WT-19 cells. Splenocytes were harvested after seven days and stained directly with CD3, CD8, MHC Db/I tetramer and MHC Kb/IV tetramer. Parallel samples were stimulated with TAg epitope I or IV for 4 hours, then stained for intracellular IFN- γ and TNF- α in addition to CD3, CD8, MHC Db/I tetramer and MHC Kb/IV tetramer. Stained samples were evaluated by flow cytometry. **(a)** Representative images of livers in tumor-free and tumor-bearing mice with or without B6-WT-19 cell immunization. Yellow arrows and values indicate tumors and tumor sizes. Left panel: wild type C57BL/6 mice; middle panel: tumor-bearing mice with tumor size $<100 \text{ mm}^3$ (early-stage tumor); right panel: tumor-bearing mice with tumor size $>400 \text{ mm}^3$ (late-stage tumor). **(b)** The mean frequency of TAg epitope I-specific CD8⁺ T cells determined by Db/I tetramer staining. **(c)** The mean frequency of TAg epitope IV-specific CD8⁺ T cells determined by Kb/IV tetramer staining. **(d)** The mean frequency of TAg peptide I-specific CD8 T cells producing both IFN- γ and TNF- α . **(e)** The mean frequency of TAg peptide IV-specific CD8 T cell producing both IFN- γ and TNF- α . ** $p < 0.01$; $n=5$; error bars represent mean $\pm$ SD.

Figure 3. Immune response of exogenous TAS TCR-I T cells in early-stage and late-stage tumor mice

TAS TCR-I T cells were isolated from the spleen and LNs of 416 mice, and labeled with CFSE. 1×10^6 naïve CFSE-labeled TCR-I T cells were adoptively transferred into tumor-free and tumor-bearing mice at early and late stage, followed by immunization with 3×10^7 B6/WT-19 cells the next day. Seven days after immunization, lymphocytes were isolated from spleens and directly stained with CD8 and Db/I tetramer or stimulated in vitro with TAG epitope I peptide for 4 hours followed by staining for intracellular IFN- γ and TNF- α in addition to CD3, CD8, and with Db/I tetramer. Samples were evaluated by flow cytometry.

(a) Representative plots showing the frequency of CSFE^{hi} (top) and CSFE-diluted (bottom) CD8⁺ T cells; (b) Cumulative data showing the frequency of total CSFE^{hi} and CSFE-diluted CD8⁺ T cells; (c) The mean frequency of IFN- γ -producing TCR-I T cells in tumor-free mice and early-stage tumor-bearing mice. (d) The mean frequency of IFN- γ -producing TCR-I T cells in tumor-free mice and late-stage tumor-bearing mice. Representative results for Fig 3c-3d are shown in **Supplementary Fig 5**. ** $p < 0.01$; n=5 mice per group; error bars represented mean $\pm$ SD.

Figure 4. Tumor cells home to local lymph nodes and circulate in the bloodstream of late-stage tumor-bearing mice

(a) The sensitivity and specificity of PCR for TAG detection. Lymphocytes were isolated from portal lymph nodes (LNs) of normal C57BL/6 and TAG-transgenic MTD2 mice. Varying numbers of lymphocytes from MTD2 mice were mixed with 10^6 lymphocytes from normal mice, and then genomic DNA was extracted from mixed lymphocytes and used as a template for PCR. **(b)** PCR detection of TAG in livers and LNs of the indicated mice. The draining portal LNs and liver were isolated from tumor-free, early-stage or late stage tumor-bearing mice. Genomic DNA was extracted from 10^6 lymphocytes or liver cells and used as templates for PCR. **(c)** Isolation and characterization of CTCs. Blood was collected from early-stage and late-stage tumor-bearing mice and CTCs enriched with a microfilter device. The enriched cells on the filters were stained by for immunofluorescence analysis with the indicated antibodies and representative images are shown.

Figure 5. The increase in the expression of PD-1 and frequency of Treg in late-stage tumor-bearing mice

Cell suspensions from spleen, liver and tumors of tumor-free mice, early-stage and late-stage tumor-bearing mice were stained for CD3, CD4, CD8, CD25, PD-1, CTLA-4, FoxP3, Gr-1 and CD11b and analyzed by flow cytometry. **(a-d)** Representative dot plots and cumulative results are presented for **(a)** the frequency of CTLA-4-expressing CD4⁺ T cells, **(b)** the frequency of PD-1-expressing CD4⁺ T cells, **(c)** the frequency of CTLA-4-expressing CD8⁺ T cells and **(d)** the frequency of PD-1-expressing CD8⁺ T cells. **(e)** Cumulative results showing the frequency of CD25⁺ and FoxP3⁺ CD4 T cells (Tregs; left) and CD11b⁺ and Gr-1⁺ cells (MDSC; right) among splenocytes. **(f)** Cumulative results showing the frequency of CD25⁺FoxP3⁺ CD4 T cells (Tregs) in liver-resident and tumor-resident lymphocytes (left) and Gr-1⁺CD11b⁺ MDSC in liver-resident and tumor-resident lymphocytes (right). Representative plots for Fig 5e-5f are shown in Supplementary Fig 6. n=3, * *p<0.01, error bars represent mean ±S.Ds.

Figure 6. Sunitinib synergizes with anti-PD-1 Ab to prevent tumor growth and activate anti-tumor immunity

(a) Experimental design of therapeutic approach in tumor-bearing mice. Size-matched tumor-bearing mice were randomly assigned to four groups and received the indicated monotherapy or combined therapy. (b) Mean tumor volume over the time course of the experiment as determined by MRI. (c) Representative images of MRI scans from the start and endpoints of the experiment. (d) Waterfall plots showing the change in tumor volume at the experimental endpoint relative to the starting tumor volume for each individual mouse. (e) Representative dot plots showing the frequency of CD25⁺FoxP3⁺ CD4⁺ T cells (Treg) among the indicated TILs. (f) Representative dot plots showing the frequency of PD-1-positive cells in gated CD8⁺ TILs. (g) Representative fold change in the frequency of CD8⁺ T cells producing IFN-γ. TILs were isolated from tumor-bearing mice given the indicated treatments and stimulated with epitope I or epitope IV peptide for 4 hours. After staining for

CD3, CD8 and intracellular IFN- γ , the frequency of CD8⁺ T cells producing IFN- γ in gated CD3⁺ T cells was determined.

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