β-Catenin Activation Promotes Immune Escape and Resistance to Anti–PD-1 Therapy in Hepatocellular Carcinoma

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ABSTRACT PD-1 immune checkpoint inhibitors have produced encouraging results in patients with hepatocellular carcinoma (HCC). However, what determines resistance to anti–PD-1 therapies is unclear. We created a novel genetically engineered mouse model of HCC that enables interrogation of how different genetic alterations affect immune surveillance and response to immunotherapies. Expression of exogenous antigens in MYC;Trp53−/− HCCs led to T-cell–mediated immune surveillance, which was accompanied by decreased tumor formation and increased survival. Some antigen-expressing MYC;Trp53−/− HCCs escaped the immune system by upregulating the β-catenin (CTNNB1) pathway. Accordingly, expression of exogenous antigens in MYC;CTNNB1 HCCs had no effect, demonstrating that β-catenin promoted immune escape, which involved defective recruitment of dendritic cells and consequently impaired T-cell activity. Expression of chemokine CCL5 in antigen-expressing MYC;CTNNB1 HCCs restored immune surveillance. Finally, β-catenin-driven tumors were resistant to anti–PD-1. In summary, β-catenin activation promotes immune escape and resistance to anti–PD-1 and could represent a novel biomarker for HCC patient exclusion.

SIGNIFICANCE: Determinants of response to anti–PD-1 immunotherapies in HCC are poorly understood. Using a novel mouse model of HCC, we show that β-catenin activation promotes immune evasion and resistance to anti–PD-1 therapy and could potentially represent a novel biomarker for HCC patient exclusion.

See related commentary by Berraondo et al., p. 1003.

INTRODUCTION Hepatocellular carcinoma (HCC) represents a major health problem, causing more than 700,000 deaths annually worldwide (1). Although HCC treatment has greatly improved over the last decades, patients with HCC diagnosed at advanced stages are ineligible for curative ablative therapies such as liver resection or transplantation. Until recently, the only FDA-approved therapy for such patients was sorafenib (2), a multikinase inhibitor that provides a 3-month survival benefit on average. In the last two years, several other multikinase inhibitors have shown efficacy in patients with advanced HCC (3–5). Lenvatinib has been approved as a first-line therapy (3), and regorafenib, an inhibitor closely related to sorafenib, is approved in second line (4). Unfortunately, these multikinase inhibitors also confer limited survival benefits. More recently, nivolumab and pembrolizumab, two PD-1 immune checkpoint inhibitors, were granted accelerated approval by the FDA for HCC treatment in second line after obtaining promising outcomes in phase II clinical trials (6, 7). The results from the nivolumab and pembrolizumab trials showed that some patients with HCC achieve unprecedented responses (6, 7). However, not all patients are sensitive, indicating the existence of mechanisms that drive resistance to anti–PD-1 therapy and highlighting the urgent need to identify biomarkers for optimal patient selection.

Cancer immunotherapy is revolutionizing the clinical management of a variety of cancers (8). Among the different immunotherapy strategies, PD-1 pathway inhibitors have provided the best clinical outcomes (9–11). Unfortunately, the clinical efficacy of PD-1 pathway inhibition as monotherapy is limited to subsets of patients, with overall response rates of 20% or less (9). In other malignancies, response rates have been significantly improved through selection of patients presenting mismatch repair deficiency (12, 13) or the combination of PD-1 pathway inhibition with other therapeutic strategies, such as CTLA4 mAbs (10), strongly supporting efforts to identify biomarkers for patient selection and novel combinatorial therapies. The general consensus is that anti–PD-1 therapies are effective in tumors that are able to trigger some level of antitumor immunity, as evidenced by the existence of CD8+ T-cell infiltrates (9). Conversely, most tumors that disrupt
antitumor immunity lack CD8+ T-cell infiltration and tend to be resistant (9). Tumor-intrinsic properties, such as mutational load (14, 15), presentation of tumor antigens (16, 17), or specific oncogenic pathways (18, 19), can greatly influence antitumor immunity and response to anti–PD-1 therapies. In melanoma, activation of β-catenin (encoded by CTNNB1; ref. 19) or PTEN deletion (18) can lead to T-cell exclusion and resistance to anti–PD-1. In HCC, two recent studies in patients have shown that β-catenin activation correlates with T-cell exclusion (20) and resistance to anti–PD-1 therapy (21). However, the mechanistic link between β-catenin activation and immune resistance has not been provided, in part due to the relative delay of the clinical trials testing immunotherapies in HCC when compared with other malignancies (such as melanoma or non-small cell lung cancer [NSCLC]), and also due to the lack of appropriate models.

Several mouse models have been generated to gain insights into the mechanisms by which tumors may subvert immune responses, but each of these has critical limitations (22, 23). For example, transplantation of primary or cultured tumor cells is commonly used, but the ectopic introduction of fully developed tumor cells bypasses the initial steps of tumorigenesis and can lead to aberrant inflammatory responses (24, 25). Carcinogen-induced models lead to robust immune responses, but the presence of multiple and heterogeneous mutations hampers the understanding of the contribution of each mutated gene to the observed phenotypes (26). Genetically engineered mouse models (GEMM) of cancer accurately recapitulate both the genetic and histopathologic progression of human disease (27), but tumors tend to be nonimmunogenic and therefore fail to reproduce the interplay between tumor cells and the immune system that is characteristic of human tumors (23). Transgenic mouse models of cancer that develop tumors spontaneously and overexpress model antigens throughout targeted organs exist, but the widespread expression of the antigens tends to induce tolerance (28), failing to recapitulate the immune responses against human tumors. Recently, Tyler Jacks’s laboratory has addressed these limitations by combining a conditional GEMM (KrasG12D;Lox-Stop-Lox/Topp53Lox-Lox) with the delivery of lentiviruses that simultaneously express Cre recombinase (which recombines the Lox sites, allowing the expression of mutant Kras and deletion of Topp53) and exogenous antigens (17, 29). The expression of exogenous antigens in mosaic tumor cells led to tumor delay as a result of tumor immune surveillance (17, 29) and formally demonstrated cancer immunoeediting in vivo (17). Although this strategy represents a technical and conceptual advancement from previous models, it is limited by the availability of existing conditional GEMMs.

In an effort to investigate the role that different genetic alterations have in HCC immune surveillance and response to immunotherapies, we have adopted a system to quickly induce autochthonous and mosaic liver tumors that harbor specific and customizable genetic alterations and varying levels of immunogenicity. The model is based on the hydrodynamic tail-vein delivery of genetic elements (30) to overexpress oncogenes (with transposon-based vectors), delete or mutate suppressor genes (with CRISPR/Cas9 vectors), and modulate immunogenicity (with exogenous antigens) specifically in hepatocytes. This model, which is amenable to rapid genetic manipulation, is technically and conceptually innovative, as it will allow us to study how different tumor-intrinsic signaling pathways affect antitumor immunity. With this model, we have shown that β-catenin activation promotes immune escape in HCC. Mechanistically, β-catenin activation led to a defective recruitment of dendritic cells (DC) and antigen-specific T cells, and as a consequence, to an impaired antitumor immune response. Reexpression of chemokine (C-C motif) ligand 5 (CCL5), a chemokine found to be downregulated in both murine and human tumors driven by β-catenin activation, restored immune surveillance. Finally, β-catenin activation conferred resistance to anti–PD-1 therapy in our murine model. We have shown that our model can be used to identify mechanisms of immune escape and resistance to anti–PD-1 that are relevant to human disease and could provide the rationale for improved patient selection and personalized cancer immunotherapies.

RESULTS
Expression of Exogenous Antigens in Murine MYC;Trp53−/− HCCs Leads to a Delay in Tumor Development

Two of the most frequently altered genes in patients with HCC are the oncogene MYC (amplified in 17% of HCCs) and the tumor suppressor Topp53 (deleted or mutated in 33% of HCCs). Their alterations frequently co-occur in patients with HCC (6.5%), suggesting cooperation (Fig. 1A). We previously showed that we can generate liver tumors resembling human HCC by performing hydrodynamic tail-vein injections of a transposon vector expressing MYC (p73-EFla-MYC), a vector expressing SB13 transposase (CMV-SB13), which is required to integrate the transposon-based vector into the hepatocyte genomic DNA, and a CRISPR/Cas9 vector expressing a single-guide RNA (sgRNA) targeting Topp53 (px330-sg-p53; ref. 31). Hydrodynamic tail-vein injections (30) allow the delivery of DNA specifically into the hepatocytes by creating an increase in blood pressure that redirects the flow of blood directly into the liver. To modulate the immunogenicity of the MYC;Trp53−/− liver tumors, we modified the transposon vector expressing MYC to also express luciferase (MYC-luc), which is mildly immunogenic (32), or a highly immunogenic version of luciferase (MYC-lucOS) that is linked to three model antigens: SIYRRYYGL (SIY), SIINFEKL (SIN; OVA257-264), and OVA323-339 (Fig. 1B; ref. 29). Hydrodynamic injection of px330-sg-p53 and CMV-SB13 in combination with MYC-luc or MYC-lucOS into 6-week-old C57BL/6 female mice led to equivalent luciferase expression in the livers measured by bioluminescence imaging at day 6, indicating similar injection efficiency and expression levels in both groups (Fig. 1C and D). Interestingly, 25 days after the injection there was a drastic reduction in luciferase signal in MYC-lucOS;sg-p53 mice, suggesting clearance of luciferase and antigen-expressing hepatocytes (Fig. 1C and D). Additional experiments demonstrated that the decrease in luciferase signal in MYC-lucOS;sg-p53 mice occurred by day 13 after the injection (Supplementary Fig. S1A). Accordingly, tumor formation was markedly delayed in MYC-lucOS;sg-p53 mice compared with MYC-luc;sg-p53 mice and was accompanied by a significant increase in survival (Fig. 1E and F). Similar effects were observed in female and male C57BL/6 mice, indicating that the phenomenon occurs
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**Figure 1.** Expression of exogenous antigens in murine MYC;Trp53−/− HCCs leads to tumor delay. A, Oncoprint of TP53 and MYC alterations in 366 patients with HCC (TCGA, provisional, December 2018, cBioPortal; ref. 61). The percentage of patients harboring the alteration is shown. Amp, amplification; del, deletion; trun, truncating; mut, mutation; infr, in-frame; miss, missense. B, Schematic of vectors injected into mice. The transposon-based vector over-expressing MYC can also express luciferase (luc) or a luciferase fused to model antigens (lucOS). C, Bioluminescence imaging 6 and 25 days after injection of vectors into representative mice. The color code for the luciferase signal is shown. D, Quantification of normalized luciferase signal 6 and 25 days after injection of vectors (n = 5 per group). Mean and SD are shown. Mann–Whitney test. Survival curves in C57BL/6 WT females (E) and males (F). Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. G, Pictures of representative livers from E and F. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. ***, P < 0.001; ****, P < 0.0001.

irrespective of sex (Fig. 1E and F). Most MYC-lucOS;sg-p53 mice did not develop any tumors within 4 months, whereas the majority of MYC-luc;sg-p53 mice presented gross liver tumors (Fig. 1G) that caused death, with a median survival of 35 to 44 days (Fig. 1E and F). Deep sequencing analysis of MYC-luc;sg-p53 livers 7 days after the injection detected 5.645% frameshift mutations at the sg-p53 target site, whereas only 0.457% indels were found in mice injected without sg-p53, and those indels were spread across the whole sequence, indicating background sequencing errors (Supplementary Fig. S1B and S1C). This confirms that px330-sg-p53 can directly generate mutations in Trp53 in the mouse liver (33). In established tumors, approximately 80% frameshift mutations and prevalence of two specific indels that produce truncated proteins were detected in tumors with sg-p53 (Supplementary Fig. S1D and S1E), suggesting selection from a single Trp53-mutated cell. In addition, transgenic MYC overexpression was confirmed in MYC;Trp53−/− HCCs when compared with normal
CD8\(^+\) T Cells Eliminate Antigen-Expressing MYC;Trp53\(^{-/-}\) HCCs

To functionally interrogate the involvement of T cells in the elimination of antigen-expressing cancer cells, we performed hydrodynamic injection of pX330-sg-p53 and CMV-SB13 in combination with MYC-luc or MYC-lucOS into 6-week-old B and T cell–deficient Rag2\(^{-/-}\) mice in C57BL/6 background (Fig. 2A and B; Supplementary Fig. S2A–S2D). The survival benefit observed in wild-type (WT) mice harboring MYC-lucOS;sg-p53 tumors was abolished in Rag2\(^{-/-}\) mice (Figs. 1E and F and 2A and B; Supplementary Fig. S2A and S2B), confirming the role of lymphocytes in eliminating antigen-expressing hepatocytes. In contrast, lack of B and T cells in Rag2\(^{-/-}\) mice had no significant effect in the development of MYC-luc;sg-p53 tumors (Supplementary Fig. S2C and S2D), indicating that the expression of antigens is critical for an effective lymphocyte-mediated immune response. Both MYC-lucOS;sg-p53 and MYC-luc;sg-p53 Rag2\(^{-/-}\) mice developed large macroscopic tumors (Fig. 2C) that caused death, with a median survival of 32 to 50 days (Fig. 2A and B; Supplementary Fig. S2A–S2D). Luciferase signal was similar between WT and immunodeficient mice three days after the

Figure 2. CD8\(^+\) T cells eliminate antigen-expressing MYC;Trp53\(^{-/-}\) HCCs. Survival curves in C57BL/6 Rag2\(^{-/-}\) females (A) and males (B). Number of mice per group is shown as well as median survival. Log-rank Mantel–Cox test. C, Pictures of representative livers from A and B. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. Survival curves in C57BL/6 WT with combined CD4\(^+\) and CD8\(^+\) T-cell depletion (D) or separate CD4\(^+\) and CD8\(^+\) T-cell depletion (E). Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. Comparisons are to control mice injected with isotype control antibodies (IgG). F, Pictures of representative livers from D and E. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. *, P < 0.05.
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The expression of antigens in the context of murine MYC;Trp53+/- HCC tumors leads to immune surveillance. However, the clearance of tumor cells was not complete in all mice, and some tumors eventually escaped the immune system (Fig. 1E and F). To identify the signaling pathways involved in the immune escape of MYC-lucOS;sg-p53 tumors, we performed RNA sequencing (RNA-seq) of bulk tumors from MYC-lucOS;sg-p53 and MYC-lucOS;sg-p53 female mice. Gene set enrichment analysis (GSEA; ref. 34) was used to evaluate functional enrichment of datasets related to different signaling pathways involved in HCC. Analysis of 188 oncogenic signatures available at MSigDB Collections (35) showed no significant differences between the two groups (Supplementary Table S1). Analysis of four HCC-specific gene signatures (36) demonstrated that a molecular class associated with CTNNB1 (β-catenin)-mutant human HCCs was significantly enriched in escaped MYC-lucOS;sg-p53 tumors (Fig. 3A; Supplementary Fig. S3A). Indeed, although the general transcriptional differences were minimal, Axin2, a direct target of β-catenin, was one of the top upregulated genes in escaped MYC-lucOS;sg-p53 tumors (Fig. 3B), suggesting that β-catenin activation may be involved in immune escape in HCC. Axin2 overexpression was confirmed in a larger set of MYC-lucOS;sg-p53 and escaped MYC-lucOS;sg-p53 tumors by qRT-PCR (Fig. 3C) and also by protein analysis (Supplementary Fig. S3B). Only one out of 22 MYC-lucOS;sg-p53 tumors presented Axin2 mRNA levels that were higher than the mean expression in escaped MYC-lucOS;sg-p53 tumors, whereas eight out of 23 MYC-lucOS;sg-p53 tumors had Axin2 levels higher than the mean (Fig. 3D).

To better understand mechanisms of immune escape in HCC, we performed RNA-seq of four additional escaped MYC-lucOS;sg-p53 tumors with low levels of Axin2 (hereafter referred to as low-Axin2-escaped tumors), which suggests they escaped through a different immune escape mechanism, and compared them to the escaped MYC-lucOS;sg-p53 tumors analyzed before (hereafter referred to as Axin2-escaped tumors; Fig. 3A and B). As expected, the molecular class associated with CTNNB1 (β-catenin)-mutant human HCCs (36) was enriched in Axin2-escaped tumors (Fig. 3E). Interestingly, a gene set related to adaptive immune response was significantly enriched in low-Axin2-escaped tumors (Fig. 3F), suggesting that there may be an association between β-catenin activation and the type of immune escape mechanism. CTNNB1 mRNA levels were unchanged (Supplementary Fig. S3C), which suggests that β-catenin activation, rather than CTNNB1 mRNA levels, is critical for its activity. To test whether β-catenin activation occurred in tumor cells, we separated tumor cells from immune cells in escaped MYC-lucOS;sg-p53 tumors by Percoll gradient centrifugation. Axin2 was found predominantly overexpressed in tumor cells in the escaped MYC-lucOS;sg-p53 tumors (Supplementary Fig. S3D). However, because stromal cells could not be separated from the bulk hepatocyte fraction, we cannot rule out that stromal cells may contribute to the increase in Axin2 levels and activation of the β-catenin pathway seen in MYC-lucOS;sg-p53 tumors. Interestingly, β-catenin activation promotes immune escape and resistance to anti–PD-1 therapy in melanoma (19) and correlates with “non-T cell–inflamed” HCC tumors (20) and resistance to anti–PD-1 therapy in patients with HCC (21), which together with our data suggests a role for β-catenin activation in HCC immune escape in a subset of HCC tumors.

β-Catenin Signaling Activation Promotes Immune Escape in HCC

CTNNB1 is frequently altered in patients with HCC (mutated in 27% to 37% of HCCs; refs. 37, 38), together with MYC amplification (Fig. 4A). To address whether or not β-catenin activation in tumor cells could promote immune escape of HCCs, we tested the effects of the expression of exogenous antigens in the context of β-catenin activation. For that, we performed hydrodynamic tail-vein injections of a transposon vector expressing activated β-catenin (CTNNB1-N90, which presents a deletion of the first 90 amino acids leading to constitutive activation; ref. 39) in combination with MYC-luc or MYC-lucOS and CMV-βgal3 into 6-week-old C57BL/6 female mice (Fig. 4B). Luciferase signal, measured by bioluminescence imaging, in the livers at day six was similar in the two groups (significantly lower in MYC-lucOS;CTNNB1 mice although within the same order of magnitude), indicating similar injection efficiency and expression levels (Fig. 4C and D). However, contrary to MYC-lucOS;sg-p53 mice, which showed a drastic reduction in luciferase signal at 25 days (Fig. 1C and D), MYC-lucOS;CTNNB1 mice showed strong luciferase signal at 27 days, indicating the presence of antigen-expressing hepatocytes and the absence of tumor cell clearance (Fig. 4C and D). Accordingly, tumor formation and survival were similar in MYC-lucOS;CTNNB1 and MYC-luc;CTNNB1 mice (Fig. 4E-G), suggesting that activated β-catenin impairs immune surveillance and promotes immune escape. Similar effects were observed in female and male C57BL/6 mice, indicating that the phenotype is not affected by sex (Fig. 4E-G). Both MYC-lucOS;CTNNB1 and MYC-luc;CTNNB1 mice developed large macroscopic tumors (Fig. 4G) that caused death with a median survival of 35 to 48 days (Fig. 2E-G), a latency comparable with MYC-lucOS;sg-p53 mice. As expected, absence of B and T cells in Rag2−/− mice had no significant impact in the tumor growth of MYC-luc;CTNNB1 and MYC-lucOS;CTNNB1 tumors (Supplementary Fig. S4A-S4C), further demonstrating...
that antigen expression in the context of MYC overexpression and β-catenin activation does not lead to immune surveillance and β-catenin activation promotes immune escape.

Luciferase signal was equivalent in MYC-luc;sg-p53, MYC-lucOS;sg-p53, MYC-luc;CTNNB1, and MYC-lucOS;CTNNB1 animals six days after the hydrodynamic injection (Supplementary Fig. S4D), excluding that differences in initial hepatocyte transfection could have an effect on the immune surveillance and immune escape observed in the MYC-lucOS;sg-p53 and MYC-lucOS;CTNNB1 mice, respectively. Furthermore, MYC-luc;sg-p53 and MYC-lucCTNNB1 mice, which are not subjected to immune pressure, presented similar median survival [35 vs. 35.5 days in females (Figs. 1E and 4E); 44 vs. 42 days in males (Figs. 1F and 4F)], indicating that the tumor growth rate was similar in both models. As expected, MYC,CTNNB1 tumors overexpressed MYC, activated β-catenin, and displayed WT p53 (Supplementary Figs. S1E and S4E). To address whether β-catenin activation is truly driving immune escape of MYC-lucOS;CTNNB1 tumors and to rule out the

Figure 3. β-catenin signaling is activated in immune-escaped HCC tumors. A, GSEA of an HCC CTNNB1 gene signature in escaped MYC-lucOS;sg-p53 tumors (n = 3 per group). NES, normalized enrichment score. B, Volcano plot representing genes according to their fold change and P-value in MYC-luc;sg-p53 escaped tumors when compared with MYC-lucOS;sg-p53 tumors. Axin2 is highlighted in red. C, Relative levels of Axin2 in MYC-luc;sg-p53 and escaped MYC-lucOS;sg-p53 tumors by qRT-PCR. Each dot represents one tumor coming from one independent mouse. Number of samples is shown. The samples used in the RNA-seq (A and B) are highlighted in blue. Mean and SD are shown. Mann-Whitney test. D, Number of cases with Axin2 levels higher (blue) or lower (black) than 2.5 (which is the mean value in MYC-lucOS;sg-p53 tumors in C). Number of samples is shown. Fisher exact test. GSEA of an HCC CTNNB1 gene signature (E) and an adaptive immune response signature (F) in Axin2-escaped MYC-lucOS;sg-p53 tumors (n = 3) or low-Axin2-escaped MYC-lucOS;sg-p53 tumors (n = 4). *, P < 0.05.
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**Figure 4.** β-catenin signaling activation promotes immune escape in HCC. A, Oncoprint of CTNNB1 and MYC alterations in 366 patients with HCC (TCGA, provisional, December 2018, cBioPortal; ref. 61). The percentage of patients harboring the alteration is shown. Amp, amplification; del, deletion; trun, truncating; mut, mutation; infr, in-frame; miss, missense. B, Schematic of vectors injected into mice. The transposon-based vector overexpressing MYC can also express luciferase (luc) or a luciferase fused to model antigens (lucOS). C, Bioluminescence imaging 6 and 27 days after injection of vectors into representative mice. The color code for the luciferase signal is shown. D, Quantification of normalized luciferase signal 6 and 27 days after injection of vectors (n = 5 per group). Mean and SD are shown. Mann–Whitney test. Survival curves in C57BL/6 WT females (E) and males (F). Number of mice per group is shown as well as median survival. Log-rank Mantel–Cox test. G, Pictures of representative livers from E and F. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. *, P < 0.05.

potential involvement of WT p53, we assessed the effect of mutating p53 in the context of MYC-lucOS;CTNNB1 tumors. Tumor formation and survival were equivalent in MYC-lucOS;CTNNB1;sg-p53 and MYC-lucOS;CTNNB1 mice (Supplementary Fig. S4F and S4G), confirming the role of β-catenin activation in driving immune escape of MYC-lucOS;CTNNB1 tumors. These results also indicate that β-catenin activation can directly promote immune escape of MYC-lucOS;sg-p53 tumors, which otherwise undergo immune surveillance (Fig. 1).

**β-Catenin Activation Impairs DC Recruitment in the Context of HCC**

There are multiple mechanisms by which cancer cells escape the immune system, involving changes in cancer and/or immune cells (8). To identify the potential changes in cancer...
cells contributing to β-catenin activation–mediated immune escape, we performed RNA-seq of bulk tumors from MYC-luc;CTNNB1 and MYC-lucOS;CTNNB1 female mice. As expected, β-catenin–driven tumors were significantly enriched in the dataset representing CTNNB1-mutant human HCCs (36) when compared with MYC-luc;sg-p53 tumors (Supplementary Table S2). In addition, levels of Axin2, a direct target of β-catenin, were higher in the β-catenin–driven tumors when compared with MYC-luc;sg-p53 tumors by qRT-PCR in a larger subset of tumors (Supplementary Fig. S5A). Moreover, luciferase transcripts were present in both groups, at similar levels, whereas transcripts corresponding to the OS region of lucOS were present in only MYC-lucOS;CTNNB1 tumors, confirming that antigen expression was not lost in the immune-escaped tumors (Supplementary Fig. S5B). High PD-L1 (CD274) has been observed in metastatic CTNNB1–mutant HCC tumor cells (40).

In our murine tumors, PD1l expression was similar in MYC-luc;sg-p53 and β-catenin–driven tumor cells (Supplementary Fig. SSC), suggesting that the immune escape observed in β-catenin–driven tumors was not due to PD1l expression in tumor cells. Interestingly, transcriptional differences between MYC-luc;CTNNB1 and MYC-lucOS;CTNNB1 tumors were negligible (156 genes overexpressed and 183 genes downregulated in MYC-lucOS;CTNNB1 tumors compared with MYC-luc;CTNNB1 tumors). The lack of changes in the “lucOS-expressing” tumors suggested that MYC-lucOS;CTNNB1 tumors may not be subjected to immune pressure, unlike MYC-lucOS;sg-p53 tumors, and that β-catenin could drive a program that completely abolishes the antitumor immune response.

To identify mechanisms of immune escape related to changes in the immune cell compartment, we performed flow cytometry analysis of the livers two weeks after the injections, a time point that already shows a decrease in luciferase signal (Supplementary Fig. S1A), in control WT, MYC-lucOS;sg-p53, and MYC-lucOS;CTNNB1 mice. The lucOS transgene leads to the expression of the model antigens SIINFEKL (SIY), SIINFEKL (SIN; OVA257-264), and OVA323-339 (Fig. 1B; ref. 29). SIINFEKL-specific CD8+ T cells were significantly more abundant in MYC-lucOS;sg-p53 livers when compared with MYC-lucOS;CTNNB1 or healthy livers (Fig. 5A). To prime antigen-specific CD8+ T cells, DCs are required. DC1 cells (DAP-1−/CD45−/lin/MHCII−CD11c−CD11b+CD24+CD103−/Axin2−/BP3−/CD8b1−/Cd3d−/Cd3e−/Cd4−/Cd8a−/Cd8b1−/BATF3−/IRF8−/Thbd−/T Cell) were absent in control WT livers (Supplementary Fig. S5A and B), two well-established targets of β-catenin pathway, harbored significantly less immune-related transcripts, suggesting that they are immune-excluded (Fig. 5G; Supplementary Table S3). Similar results were obtained by immunofluorescence staining for the T-cell marker CD3 in MYC-lucOS;sg-p53 and MYC-lucOS;CTNNB1 livers after 2 weeks, recapitulating the flow cytometry results (Supplementary Fig. S5E and S5F).

We also assessed the transcriptional profiles of 360 samples from patients with HCC [liver hepatocellular carcinoma (LIHC), available at The Cancer Genome Atlas (TCGA); ref. 37]. As expected, CTNNB1-mutant samples (97/360, 27.2%) were significantly enriched in the dataset representing CTNNB1–mutant HCCs (36) when compared with CTNNB1 WT samples (Supplementary Table S2). In fact, expression of AXIN2 and GLUL, two well-established targets of β-catenin, was significantly higher in CTNNB1-mutant samples (Fig. 5H; Supplementary Table S4). Most importantly, CTNNB1-mutant samples presented significantly reduced expression of DC markers (BATF3, IRF8, THBD), T-cell markers (CD3D, CD3E, CD4, CD8A), and the exhaustion marker PDCD1 (PD-1; Fig. 5H; Supplementary Table S3), suggesting that CTNNB1–mutant HCCs exhibit immune exclusion. In fact, in a cohort of 59 HCC patient samples, nuclear staining of β-catenin was associated with significantly lower numbers of CD8+ T cells in the tumors (Fig. S1 and J), and in another cohort of 216
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Figure 5. β-catenin impairs DC recruitment in the context of HCC. Quantification of the percentage of SIINFEKL-specific CD8+ T cells (A) or number of DC1 DCs (B) in the livers of the corresponding mice (n = 5–6 per group). Representative of three independent experiments. N, normal liver; sg-p53, MYC-lucOS;sg-p53; Control, noninjected; KO, Batf3−/− females harboring MYC-lucOS;sg-p53 tumors. Batf3−/− mice are represented with a dotted line. Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. D, Pictures of representative livers from C. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. Quantification of the number of DC1 dendritic cells (E) or the percentage of SIINFEKL-specific CD8+ T cells (F) in the livers of the corresponding mice (n = 5 per group). sg-p53, MYC-lucOS;sg-p53; Control, noninjected; KO, Batf3−/−. Mean and SD are shown. ANOVA test. Heat map showing the average expression values of different genes in murine tumors (G) and human TCGA tumors (H). Colors are adjusted for each row and for each group comparison from high (red) to low (blue). luc p53, MYC-luc;sg-p53 (n = 3); lucOS p53, MYC-lucOS;sg-p53 (n = 3); low Axin2, low-Axin2-escaped MYC-lucOS;sg-p53 (n = 4); luc CTNNB1, MYC-luc;CTNNB1 (n = 7); lucOS CTNNB1, MYC-lucOS;CTNNB1 (n = 5); CTNNB1 WT, wild-type (n = 263); CTNNB1 mut, mutant (n = 37); CTNNB1 low (n = 120); CTNNB1 inter, intermediate (n = 120); CTNNB1 high (n = 120). Samples were stratified depending on CTNNB1 status as WT or mutant, or CTNNB1 mutant HCC gene signature enrichment levels (tertiles). Number of CD8+ T cells in tumor and peritumor areas in HCC patient samples (n = 59), which were classified as having membrane (mb; n = 37) or nuclear (nuc; n = 22) staining for β-catenin protein. Mean and SD are shown. Mann–Whitney test. I, Representative pictures of the stainings for CD8 and β-catenin summarized in I. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
patients with HCC, those with enrichment of CTNNB1-mutant HCC signature (36) were associated with a significant decrease in immune cell infiltration assessed by hematoxylin and eosin staining (Supplementary Fig. S5L). To test the importance of β-catenin pathway activation levels on the immune escape phenotype, we stratified the 360 patients with HCC according to their level of enrichment of the dataset representing CTNNB1-mutant HCCs (ref. 36; low, first tertile; intermediate, second tertile; high, third tertile). As observed in the murine tumors (Fig. 5G; Supplementary Table S3), samples from patients with HCC with intermediate and high activation of the β-catenin pathway presented less immune cell transcripts than samples in the low activation group, further suggesting that β-catenin pathway activation levels have an impact on the extent of immune exclusion. Taken together, the immune escape driven by β-catenin activation is mediated by a defect in DC recruitment, which in turn impairs the subsequent antitumor immune response, in both murine and human HCCs.

**CCL5 Expression Restores Immune Surveillance in β-Catenin-Driven HCCs**

To identify mechanisms explaining the defective DC activity in the context of β-catenin activation in HCC, we explored the expression of chemokines in MYC-luc;gp53 tumors (control; not exposed to immune pressure) and β-catenin-driven tumors (MYC-lucOS;CTNNB1 and MYC-luc;CTNNB1). Six chemokines (CCL5, CXCL1, CCL20, CCL28, CCL17, and CXCL10) out of 34 chemokines quantified by RNA-seq were significantly downregulated in β-catenin-driven tumors (there were no chemokines upregulated; Fig. 6A; Supplementary Fig. S6A; Supplementary Table S5). Among these, CCL5, CCL20, and CXCL1 were also downregulated in human CTNNB1-mutant HCC samples (Supplementary Fig. S6B; Supplementary Table S6). Because CCL5 has been shown to affect different immune cells, including DCs (42), we decided to focus on CCL5. We further confirmed the low levels of CDS in murine β-catenin-driven tumors by qRT-PCR of an independent subset of MYC-luc;gp53 tumors (control; not exposed to immune pressure) and β-catenin-driven tumors (MYC-lucOS;CTNNB1 and MYC-luc;CTNNB1; Supplementary Fig. S6C). CDS levels were similar in MYC-luc;gp53 and Axin2-escaped MYC-lucOS;gp53 tumors. However, compared with low-Axin2-escaped MYC-lucOS;gp53 tumors, CDS levels were slightly lower (although not statistically significant) in Axin2-escaped MYC-lucOS;gp53 tumors (Supplementary Fig. S6D), which present intermediate activation of the β-catenin pathway (Fig. 5G). Similarly, in TCGA HCC patient samples, there was a graded decrease in CCL5 expression with increasing β-catenin activation: Tumors with the lowest activation of the β-catenin pathway showed higher CCL5 expression than tumors with intermediate activation of the β-catenin pathway (although again not statistically significant; Fig. 6B).

However, tumors with high activation of the β-catenin pathway displayed significantly less CCL5 than tumors with low activation of β-catenin, further supporting the link between β-catenin and CCL5 expression (Fig. 6B). CDS was found to be expressed in both immune and tumor cells in MYC-luc;gp53 mice (Supplementary Fig. S6E). Interestingly, CDS expression increased significantly in MYC-lucOS;gp53 livers between 7 and 21 days (Supplementary Fig. S6F), replicating the timing of immune cell infiltration and suggesting that CCL5 may be a critical mediator of the antitumor immune response.

To test whether or not CCL5 overexpression in tumor cells could somehow elicit an antitumor immune response and revert the immune escape observed in β-catenin–driven tumors, we tested the effects of the expression of model antigens in the context of simultaneous β-catenin activation and CCL5 overexpression. For that, we cloned a CDNA encoding for CD5 in the same vector as CTNNB1-N90 (Fig. 6C). We then performed hydrodynamic tail-vein injections of this vector in combination with MYC-luc or MYC-lucOS and CMV-SB13 into 6-week-old C57BL/6 female mice. Tumor formation and survival were significantly delayed in MYC-lucOS;CTNNB1-CD5 mice when compared with MYC-luc;CTNNB1-CD5 mice, suggesting that CCL5 expression restores immune surveillance in the context of β-catenin activation and antigen expression (Fig. 6D and E). Mechanistically, expression of CCL5 in β-catenin–driven tumors led to a significant increase in the levels of DC1 (DAPI+CD45+Lin+MHCIIC-D11c+CD24+CD103+CD11b+; Fig. 6F) and antigen-specific CD8+ T cells (Fig. 6G) compared with healthy livers, which could potentially explain the restoration of immune surveillance. As expected, injection of anti-CD4 and anti-CD8 antibodies into MYC-lucOS;CTNNB1-CD5 WT mice led to a decrease in survival when compared with mice treated with control antibodies (Fig. 6H and I). This is similar to the effects seen in DC-deficient Batf3−/− mice (Fig. 6J–L) and further confirms the role of CCL5 in mounting an antitumor immune response. In conclusion, CCL5 expression is downregulated in β-catenin-driven murine and human HCCs, and CCL5 reexpression leads to the restoration of the immune surveillance program.

**β-Catenin Signaling Activation Confers Resistance to Anti–PD-1 Therapy in HCC**

Nivolumab and pembrolizumab, two anti–PD-1 inhibitors, have recently been approved by the FDA for second-line therapy in patients with advanced HCC (6, 7). To test the therapeutic relevance of β-catenin–driven immune escape in response to anti–PD-1, we treated our novel mouse models of HCC with blocking mAbs against murine PD-1. Of note, because most MYC-lucOS;gp53 mice do not develop tumors (Fig. 1E–G), a higher dose of vector DNA was used to force tumor formation. Mice harboring MYC-lucOS;gp53 tumors were responsive to anti–PD-1 treatment (Fig. 7A). In contrast, mice harboring MYC-lucOS;gp53 tumors did not respond (Fig. 7B), demonstrating that expression of tumor antigens is a requirement for responding to anti–PD-1 therapy. In the case of β-catenin–driven tumors, neither MYC-lucOS;CTNNB1 or MYC-luc;CTNNB1 models were responsive to anti–PD-1, proving that β-catenin activation promotes resistance to immunotherapy in our models (Fig. 7C and D).

The association between β-catenin activation and resistance to anti–PD-1 therapy has been observed in patients with HCC (21): None of the 10 patients with HCC with activating mutations in CTNNB1 had response to anti–PD-1 or anti–PD-L1 therapy whereas 50% of CTNNB1 WT patients had a response. To test this further, we collected tumor specimens from 15 patients with HCC treated with nivolumab at Mount Sinai Hospital. Overall, 6 (40%) patients responded to anti–PD-1 therapy, with a median survival time of 22.2 months,
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Figure 6. CCL5 expression restores immune surveillance in β-catenin–driven HCCs. A, Venn diagram displaying the chemokines differentially expressed in mice or human liver tumors. The number of samples for each dataset is included. Red, significantly upregulated; blue, significantly downregulated. The intersection shows the chemokines dysregulated in both datasets. B, Expression of CCL5 in 360 human HCC samples (LIHC, liver hepatocellular carcinoma, from the TCGA). Box and whisker plot, with the central line representing the median, the ends of the box representing the upper and lower quartiles, and the whiskers extending to the highest and lowest observations. Mann–Whitney test. n CTNNB1 signature low (n = 360) (34 chemokines expressed in mice or human liver tumors. The number of samples for each dataset is included. Red, significantly upregulated; blue, significantly downregulated. The intersection shows the chemokines dysregulated in both datasets. C, Schematic of vectors injected into mice. The transposon-based vector overexpressing CTNNB1 also expresses Ccl5. D, Survival curves in C57BL/6 WT females. Number of mice per group is shown as well as median survival. Log-rank Mantel–Cox test. E, Pictures of representative livers from C, normal liver; N, CTNNB1–/–; IgG, IgG control; aCD4/aCD8, aCD4/aCD8 depletion. F, Survival curves in C57BL/6 WT males with combined CD4+ and CD8+ T-cell depletion. Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. G, % Survival. H, Survival curves in C57BL/6 WT females with combined CD4+ and CD8+ T-cell depletion. Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. I, Pictures of representative livers from F. J and K, Survival curves in C57BL/6 WT or Batf3–/– males (J) and females (K). Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. L, Pictures of representative livers from K. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
and 9 (60%) patients did not respond, with a median survival of 6.2 months ($P = 0.034$; Supplementary Fig. S7A). Of note, 3 patients harbored CTNNB1 mutations, two being nonresponders and one being a responder (Fig. 7E and F). Because of the small sample size (power calculation of sample size indicates that at least 89 patients would be needed), we were not able to establish statistical significance. Nevertheless, our data together with the previously published study (21) suggest a role for β-catenin in promoting resistance to anti–PD-1 therapy in HCC.

**DISCUSSION**

In this study, we have demonstrated that β-catenin activation in HCC tumor cells is an important mechanism of immune escape that confers resistance to anti–PD-1 therapies. The use of mAbs directed against inhibitory receptors on immune cells, known as immune checkpoint blockade, has aroused tremendous enthusiasm among clinicians, scientists, and patients (9). In particular, mAbs targeting PD-1/ PD-L1 have shown remarkable antitumor activity in numerous malignancies (11, 43–45), leading to their regulatory approval (9). However, despite the unprecedented efficacy of these agents in some patients, the lack of response in the majority emphasizes the pressing need to identify biomarkers that can select the patients that are most likely to benefit from therapy. In other malignancies, mismatch repair deficiency (12, 13), mutations in the SWI/SNF chromatin remodeling complex (46–48), or ADAR1 mutations (49) sensitize tumors to respond to immunotherapies. In contrast, β-catenin activation (19), PTEN deletion (18), or JAK2 mutations (16) lead to resistance to immunotherapies. Similar studies in HCC have been missing, in part due to the relative delay of the clinical trials testing immunotherapies when compared with other malignancies, such as melanoma or NSCLC. A comprehensive transcriptional
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analysis of HCC patient samples has previously found a correlation between CTNNB1 mutation and T-cell exclusion (20), suggesting that β-catenin activation could be involved in immune escape and resistance to immunotherapies in patients with HCC. This correlation between CTNNB1 mutation and T-cell exclusion has been validated across a large set of human cancers (50). Furthermore, in a small cohort of patients with HCC, alterations in the β-catenin pathway correlated with lack of response to anti-PD-1 or anti-PD-L1 therapies (21). Here, by using a novel mouse model of HCC, we have functionally shown that β-catenin activation leads to immune exclusion and resistance to anti-PD-1 therapy, which emphasizes the utility of our models to identify processes that are relevant to human disease. One limitation in HCC clinical research is that tumor biopsies are not recommended for patients with advanced HCC (51). A change in the clinical guidelines may be needed to enable liver biopsies in patients with advanced HCC to facilitate the identification of biomarkers of response and implement biomarker-guided therapies.

Mechanistically, we have demonstrated that β-catenin activation in HCC tumor cells impaired recruitment of CD103+ DCs, which are critical cells in mounting an effective antitumor immune response (52). This defective recruitment of DCs in turn impaired the presence of antigen-specific CD8+ T cells in the liver, further confirming the reduced immune surveillance. Interestingly, at an early time point, CTNNB1-mutant HCC tumors presented CD8+ T cells that were not antigen-specific and could be bystander T cells (53, 54). However, in murine and human CTNNB1-mutant established tumors, T cells were rare, consistent with an immune exclusion phenotype. Moreover, transcriptional analysis of HCC patient samples revealed that transcripts related to DCs and T cells were significantly downregulated in CTNNB1-mutant HCC tumors when compared with CTNNB1 WT tumors, extending our findings to the human setting. A similar observation has been made in melanoma, where β-catenin activation also leads to a defective recruitment of CD103+ DCs (19). In the melanoma study, the reduced recruitment of CD103+ DCs into the tumor microenvironment could partially be explained by a defective production of the chemokine CCL4. In our murine HCC model and in HCC patient samples, chemokine CCL4 expression levels were unchanged between CTNNB1-mutant and CTNNB1 WT tumor samples. Instead, we found a significant reduction in the levels of chemokines CXCL1, CCL20, and CCL5 in CTNNB1-mutant tumors in both murine and human CTNNB1-mutant samples. Because CCL5 could potentially affect DCs (42), we decided to further pursue the effect of CCL5 on immune surveillance. Indeed, overexpression of chemokine CCL5 in β-catenin-driven HCC cells led to a higher recruitment of CD103+ DCs, antigen-specific CD8+ T cells, and restoration of immune surveillance, demonstrating its causal role. Restoration of intratumor DCs by intratumor delivery of FLT3 ligand-induced bone marrow-derived DCs also had an anti-tumor effect in melanoma (19). It is striking that in different tumor types, the same signaling pathway, β-catenin activation, elicits a similar mechanism although mediated by different chemokines. It is also possible that additional chemokines and secreted molecules may be involved in the recruitment of DCs in both settings. Nevertheless, therapeutic strategies that promote DC recruitment (55) could improve the response of CTNNB1-mutant tumors to anti-PD-1 therapy.

In HCC, around one third of the patients present activating mutations in CTNNB1 and could potentially be resistant to anti-PD-1 therapies (56). So far, the clinical trials testing nivolumab (6) and pembrolizumab (7) have demonstrated that only around 15% to 20% of patients with HCC exhibit an objective response to these therapies. This suggests that other mechanisms of immune resistance beyond β-catenin activation exist. In fact, in our mouse model, less than 50% of the immune-escaped HCC tumors presented β-catenin activation (as measured by an increase in the expression levels of β-catenin target Axin2). Characterization of the remaining tumors has shown that escaped murine liver tumors, which present abundant immune-related transcripts, can escape through different mechanisms. In addition, the temporal study of MYC-ΔucOSggs53 tumor cells by single-cell RNA-seq may shed light on the initial changes occurring in tumor cells subjected to immune pressure and undergoing immune surveillance. Single-cell RNA-seq may also enable better establishment of the changes occurring in different cell compartments and the contribution of each compartment to immune escape. Moreover, it is possible that mutations co-occurring with CTNNB1 mutation may modify the effect that β-catenin activation has in antitumor immunity. In addition, the levels of activation of the β-catenin signaling pathway may affect the phenotype of immune escape. Additional studies will be needed to identify distinct mechanisms of resistance to immunotherapies and refine the set of mutations that cooperate with CTNNB1 mutation to confer resistance. To address this, it will be critical to combine mechanistic studies in mice with the analysis of HCC patient samples.

To understand the role that different genetic alterations in HCC tumor cells have in immune surveillance and response to immunotherapies, we have generated a novel mouse model of HCC. The model is based on the hydrodynamic tail-vein delivery (30) of genetic elements encoding oncogenes. CRISPR targeting tumor suppressor genes, and exogenous antigens. A similar approach, comparing tumor formation in the absence or presence of exogenous antigen expression, was used to study immune surveillance in lung cancer (29) and to demonstrate immunoediting in the context of sarcoma (17). A recent study in HCC performed hydrodynamic injection of mutant NRAS, AKT, and exogenous antigens to demonstrate that antigen-specific T cells undergo exhaustion (57). The strength of our approach is that we can compare the effect that the expression of exogenous antigens has in the context of different genetic alterations, which, coupled with the use of both immunocompetent and immunodeficient mouse models, can lead to fundamental discoveries. For example, performing experiments in the absence or presence of antigens has enabled us to demonstrate that antigen expression in the context of MYC;Trp53−/− liver tumors leads to immune surveillance. This strong antitumor immune response is driven by antigen expression and not by the loss of p53, because elimination of immune cells in mice harboring MYC;Trp53−/− liver tumors that do not express antigens has no effect. Similarly, antigen expression is critical for responding to anti-PD-1 immunotherapy because mice harboring MYC;Trp53−/− liver tumors that do not express antigens are not responsive to
the therapy. The fact that the model antigens are linked to luciferase allows monitoring of tumor growth and immune responses over time by simply performing bioluminescence imaging. However, because the antigens are genetically linked to the driving oncogene, MTC, it is likely that there is a selective pressure against the loss of the antigens. The advantage of this may be that the system allows the study of mechanisms of immune escape different from the loss of antigens. It will be interesting to study how HCC-specific tumor antigens, such as α-fetoprotein or glypican 3 (58), instead of model antigens, affect mechanisms of immune surveillance in mice. The benefit of using model antigens, such as the ones used in our study, is that they have the potential to elicit strong immune responses that can be overcome only by bona fide immune escape mechanisms. The temporal control of the expression of the antigens by using inducible systems, uncoupled from the expression of the driving oncogene, may better recapitulate tumor evolution and immune responses. Finally, HCC arises in the context of underlying liver disease. It will be critical to test how different types of liver damage (viral, alcohol-mediated, dietary), which can be easily combined with our model, affect response to immunotherapies.

In conclusion, we provide a novel mouse model of HCC that can be used to identify mechanisms of immune escape and resistance to immunotherapies that are relevant to human disease. This model represents a paradigm of personalized mouse model of HCC that recapitulates immune surveillance and allows interrogation of the role of virtually any genetic alteration in antitumor immunity. With this model, we have found that β-catenin activation promotes immune escape and resistance to anti–PD-1 therapies in HCC. By dissecting the underlying biology, we also propose a mechanism to restore immune surveillance in β-catenin-driven tumors. Finally, our results suggest that CTNNB1 mutational status could be used as a biomarker for patient exclusion. The identification of additional tumor-intrinsic signaling pathways that disrupt antitumor immunity and affect response to anti–PD-1 by using our novel mouse model may help optimize patient selection.

METHODS

Vector Design and Use

To generate the pT3-EF1a-MYC-ires-luciferase (MYC-luc) vector, the pT3-EF1α-MYC plasmid was opened with Pmel restriction enzyme, the “ires-luciferase” sequence was PCR-amplified from pMSCV-ires-luciferase, and the cloning of the “ires-luciferase” fragment into the linearized pT3-EF1α-MYC was performed by using In-Fusion HD Cloning Plus (Takara Bio). The pT3-EF1α-MYC-ires-luciferase-OS (MYC-lucOS) vector was generated by In-Fusion cloning of the Pmel-linearized pT3-EF1α-MYC vector, the “ires-luciferase” fragment, and the “OS” fragment, which was amplified from LentivLucOS vector by PCR. To generate the pT3-EF1α-CTNNB1-ires-luciferase (CTNNB1-luc) vector, the pT3-EF1α-NRAS-ires-GFP plasmid digested with XbaI and EcoRV restriction enzymes was used as a donor vector. The “CTNNB1,” “ires,” and “GFP” sequences were PCR-amplified from pT3-N90-CTNNB1, the pT3-EF1α-NRAS-ires-GFP, and the pMD-mCD5 plasmids, respectively, and the cloning of the three fragments into the donor vector was performed by In-Fusion cloning. The CMV-SB13, pT3-EF1α-NRAS-ires-GFP, and pMCSV-ires-luciferase plasmids were kindly provided by Dr. Scott Lowe (Memorial Sloan Kettering Cancer Center, New York, NY). The pT3-EF1α-MYC and pT3-N90-CTNNB1 (Addgene plasmid #31785) were a kind gift from Dr. Xin Chen (University of California, San Francisco, CA). Lenti-LucOS (Addgene plasmid #22777) was a gift from Dr. Tyler Jacks. The full-length cDNA of CTNNB1 was obtained from Sino Biological (plasmid reference: MG508022-M). The px330-ires-p53 was previously published and validated (31). The px330 vector was a gift from Feng Zhang (Addgene plasmid #42230). All constructs were verified by nucleotide sequencing and vector integrity was confirmed by restriction enzyme digestion. Each new vector will be made available through Addgene.

Hydrodynamic Tail-Vein Injection

A sterile 0.9% NaCl solution/plasmid mix was prepared containing DNA. We prepared 11.4 μg of pT3-EF1α-MYC-ires-luciferase-OS (MYC-luc), 12 μg of pT3-EF1α-MYC-ires-luciferase-OS (MYC-lucOS), 10 μg of pT3-N90-CTNNB1 (CTNNB1), 27 μg of pT3-EF1α-CTNNB1-ires-Cd5 (CTNNB1-Cd5), 10 μg of px330-ires-p53 (ires-p53), and a 4:1 ratio of transposon to SB13 transposase-encoding plasmid dissolved in 2 mL of 0.9% NaCl solution and injected 10% of the weight of each mouse in volume. Because two independent “hits” are required for tumor formation in C57BL/6 mice (30), only those hepatocytes that receive the three plasmids (transposon-based, transposase, and CRISPR-based) will have the potential to form tumors. We also show that a single hepatocyte can take up to four plasmids, as shown in MYC-lucOS/CTNNB1-ires-p53 tumors. For the anti–PD-1 experiment, we used 13 μg of pT3-EF1α-MYC-ires-luciferase-OS, 13 μg of px330-ires-p53 (ires-p53), and a 4:1 ratio of transposon to SB13 transposase-encoding plasmid per 2 mL. Mice were injected with the 0.9% NaCl solution/plasmid mix into the lateral tail vein with a total volume corresponding to 10% of body weight in 5 to 7 seconds. Vectors for hydrodynamic delivery were produced using the QIAGEN plasmid PheMega kit (QIAGEN). Equivalent DNA concentration between different batches of DNA was confirmed to ensure reproducibility among experiments.

Mice

Different batches of WT C57BL/6 mice were purchased from Envigo and were used for the treatment experiments (with mAbs) or for flow cytometry experiments. Rag2−/− and Batf3−−/− mice were WT C57BL/6 mice purchased from Jackson and bred at Icahn School of Medicine at Mount Sinai (ISMMs). Controls for Rag2−/− and Batf3−−/− mice were WT C57BL/6 mice purchased from Jackson and bred at ISMMs. All mouse experiments were approved by the ISMMs Animal Care and Use Committee (protocol number IACUC-2014-0229). Mice were maintained under specific pathogen-free conditions, and food and water were provided ad libitum. All animals were examined prior to the initiation of the studies to ensure that they were healthy and acclimated to the laboratory environment. All experiments were performed with 6- to 8-week-old mice, and both males and females were used in most experiments (analyzed separately). Once the animals were sacrificed, livers were collected, formalin-fixed and paraffin-embedded, frozen, or embedded in OCT (Tissue Tek).

Deep Sequencing of CRISPR-Modified Trp53 Locus

The genomic region of Trp53 targeted by irs-p53 was PCR amplified using Platinum SuperFi (Invitrogen) high-fidelity DNA polymerase and PCR purified. The primers used were 5′-AAGGCTAAGGGGTTT GTTTG-G (forward) and 5′-GATACATGTTTAGGGATG-3′ (reverse). Libraries were made from 500 ng of the PCR products using the Nextera protocol and sequenced on Illumina MiSeq (250 base pair paired-end). Data were processed according to standard Illumina sequencing analysis procedures. The raw Illumina reads were checked for adapters and quality via FastQC. The raw illumina sequence reads were trimmed of their adapters and nucleotides with poor quality using Trimmomatic v. 0.36. Paired sequence reads were then merged to form a single sequence if the forward and reverse reads were able...
to overlap. The merged reads were aligned to the reference sequence using bwa version 0.7.12, and variant detection was performed using GENEWIZ proprietary Amplicon-EZ program. Two to four biological replicates were sequenced for in vivo liver samples.

**Treatments**

Treatments were initiated one week after the hydrodynamic delivery of the plasmids, a time point that already presents malignant tumor cells. For the experiments with anti–PD-1 mAbs, three doses of either anti–PD-1 (200 μg, clone RMPI-1, BioXcell) or IgG (200 μg, IgG2b, clone LTF-2, BioXcell) were given intraperitoneally at days 7, 9, and 11. For the T-cell depletion experiments, mice were injected intraperitoneally with anti–CD4 (200 μg, clone GK1.5, BioXcell), anti–CD8 (200 μg, clone 24-3, BioXcell), or IgG (200 μg, IgG2b, clone LTF-2, BioXcell) at days 7, 9, 11, and 13, and then once weekly until the end of the experiment. For the combined depletion of CD4+ and CD8+ T cells, 200 μg of each antibody were used and 400 μg of IgG.

**Luciferase Detection**

In vivo bioluminescence imaging was performed using an IVIS Spectrum system (Caliper LifeSciences, purchased with the support of NCRR S10-RR026561-01) to quantify liver tumor burden before being evenly assigned to various treatment study cohorts. Mice were imaged 5 minutes after intraperitoneal injection with fresh α-luciferin (150 mg/kg, Thermo Fisher Scientific). Luciferase signal was quantified using Living Image software (Caliper LifeSciences). Normalized luciferase signal was calculated by subtracting the background signal. Each treatment cohort had equivalent average luciferase signal. Those mice with a luciferase signal a log of magnitude ground signal. Each treatment cohort had equivalent average lucif-

**RNA-seq and Analysis**

RNA was poly-A selected, and multiplexed RNA-seq libraries were prepared using the TruSeq RNA Sample Preparation kit (Illumina) according to the manufacturer’s instructions at the ISMMS Genomics Core. The libraries were quantified using the Qubit Broad Range kit (Thermo Fisher Scientific) and sequenced using the Illumina HiSeq 4000 system (SR100). The RNA-seq data was analyzed using Bbasepair software (www.basepairtech.com) with a pipeline that included the following steps. Reads were aligned to the transcriptome derived from UCSC genome assembly mm10 using Tophat2 with default parameters. Read counts for each transcript were measured using featureCounts (59). Differentially expressed genes were determined using DESeq2 (60) and a cutoff of 0.05 on adjusted t value. Read counts for each transcript were measured using featureCounts (59). Differentially expressed genes were determined using DESeq2 (60) and a cutoff of 0.05 on adjusted t value. Read counts for each transcript were measured using featureCounts (59). Differentially expressed genes were determined using DESeq2 (60) and a cutoff of 0.05 on adjusted t value.

**Human HCC Sample Analysis**

MYC, TP53, and CTNNB1 genomic alterations in patients with HCC (n = 366) were obtained from the cBioPortal (61) TCGA dataset. Gene expression profiling of a total of 360 human samples was extracted from the TCGA (December 2018). Samples were stratified depending on CTNNB1 status as WT or mutant, or CTNNB1-mutant HCC gene signature enrichment levels (in tertiles). Mann-Whitney test was performed to test for differences in gene expression values on the log scale.

**Patient Cohort and Evaluation of Treatment Response**

Patients receiving nivolumab at ISMMS were eligible to be enrolled in the study if they had a confirmed histologic diagnosis of HCC and viable tumor tissue (either biopsy or archival sample) prior to the start of immunotherapy. Once local Institutional Review Board (IRB) approval was granted, written informed consent for tumor profiling was obtained from each patient on a retrospective protocol (IRB number 17-01728) in accordance with the Belmont Report. Initial diagnosis of HCC was made following the clinical practice guidelines from the European Association for the Study of the Liver (62). All included patients presented an advanced (BCLC-C) or intermediate (BCLC-B) stage with prior progression to surgery and/or locoregional therapies at the moment of immunotherapy initiation. Nivolumab was administered at a dose of 240 mg every 2 weeks and was continued until toxicity, progression, or death, according to the treating physician. Assessment of response was conducted at least 3 months after treatment initiation and performed by mRECIST criteria (63). Treatment response was defined as follows: complete response (CR); disappearance of any intratumoral arterial enhancement in all target lesions; partial response (PR); at least a 30% decrease in the sum of diameters of viable (enhancement in the arterial phase) target lesions, taking as reference the baseline sum of diameters of target lesions; progression disease (PD); an increase of at least 20% in the sum of the diameters of viable (enhancing as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started; stable disease (SD); any cases not qualifying for either PR or PD). The electronic medical records were reviewed to extract information on patient’s gender, age, race, etiology, date of diagnosis, specimen location (liver, local recurrence, or extraphepatic metastasis), extent of disease, treatment history, type, number and dates of systemic therapy with radiographic response, date of progres-

**Statistical Analysis**

Data are expressed as mean ± SD. Statistical significance was determined using Mann–Whitney U test (when n < 10 or nonnormal distribution) or Student t test (n > 10 and normal distribution). For comparisons of more than two groups, we used ANOVA test. For paired comparisons, we used the Wilcoxon test. For frequency comparisons, we utilized the χ2 test. Group size was determined on the basis of the results of preliminary experiments, and no statistical method was used to predetermine sample size. Group allocation for treatments was performed to ensure equivalent luciferase signal, and outcome assessment was not performed in a blinded manner. The differences in survival were calculated using the Kaplan–Meier test. GraphPad Prism 6 software was used to create the graphs and for the statistical analysis. Significance values were set at *P < 0.05, **P < 0.01, ***P < 0.001.

The rest of material and methods can be found in Supplementary Methods.

**Disclosure of Potential Conflicts of Interest**

A. Villanueva is a consultant/advisory board member for Guidect, Fujifilm, Exact Sciences, Nucleix, NGM Pharmaceuticals, and Exelixis. J.M. Llovet reports receiving commercial research grants from Bayer Healthcare Pharmaceuticals, Eisai Inc., Bristol-Myers Squibb, and IPSEN, and is a consultant/advisory board member for Eli Lilly, Bayer Healthcare Pharmaceuticals, Navigant, Leerink Swann LLC, Midatech Ltd., Fortress Biotech Inc., Spring Bank Pharmaceuticals, Nucleix, Can-Fite Biopharma, Bristol-Myers Squibb, Eisai Inc., Celsion, Exelixis, Merck, Blueprint, Ipsen, and Glycostat. A. Lujambio reports receiving commercial research grants from Pfizer and Genentech and has received speakers bureau honoraria from Exelixis. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: M. Ruiz de Galarreta, A. Lujambio Development of methodology: M. Ruiz de Galarreta, K.E. Lindblad, M. Casanova-Acebes, A. Lujambio

**RESEARCH ARTICLE**

β-Catenin Promotes Immune Resistance in Liver Cancer

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