Blocking the CCL2–CCR2 Axis Using CCL2-Neutralizing Antibody Is an Effective Therapy for Hepatocellular Cancer in a Mouse Model

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Abstract

Hepatocellular carcinoma, a deadly disease, commonly arises in the setting of chronic inflammation. C-C motif chemokine ligand 2 (CCL2/MCP1), a chemokine that recruits CCR2-positive immune cells to promote inflammation, is highly upregulated in hepatocellular carcinoma patients. Here, we examined the therapeutic efficacy of CCL2–CCR2 axis inhibitors against hepatitis and hepatocellular carcinoma in the miR-122 knockout (a.k.a. KO) mouse model. This mouse model displays upregulation of hepatic CCL2 expression, which correlates with hepatitis that progress to hepatocellular carcinoma with age. Therapeutic potential of CCL2–CCR2 axis blockade was determined by treating KO mice with a CCL2-neutralizing antibody (nAb). This immunotherapy suppressed chronic liver inflammation in these mice by reducing the population of CD11b+Gr1+ inflammatory myeloid cells and inhibiting expression of IL6 and TNFα in KO livers. Furthermore, treatment of tumor-bearing KO mice with CCL2 nAb for 8 weeks significantly reduced liver damage, hepatocellular carcinoma incidence, and tumor burden. Phospho-STAT3 (Y705) and c-MYC, the downstream targets of IL6, as well as NF-κB, the downstream target of TNFα, were downregulated upon CCL2 inhibition, which correlated with suppression of tumor growth. In addition, CCL2 nAb enhanced hepatic NK-cell cytotoxicity and IFNγ production, which is likely to contribute to the inhibition of tumorigenesis. Collectively, these results demonstrate that CCL2 immunotherapy could be an effective therapeutic approach against inflammatory liver disease and hepatocellular carcinoma. Mol Cancer Ther; 16(2): 312–22. ©2016 AACR.

Introduction

Hepatocellular carcinoma is the most common liver cancer and the second leading cause of cancer-related deaths worldwide (1, 2). The incidence of hepatocellular carcinoma has tripled in the United States because of the precipitous increase in nonalcoholic fatty liver disease and hepatitis C virus (HCV) infection in the past 20 years (3, 4). Furthermore, sorafenib, the only FDA-approved drug for advanced hepatocellular carcinoma extends overall survival by only 2.8 months (5). Recent clinical trials have shown that it is ineffective as an adjuvant therapy after resection or ablation of the tumor (6). Thus, there is an urgent need to develop novel therapeutic strategies for the treatment of hepatocellular carcinoma.

miR-122 is the most abundant liver-specific miRNA in vertebrates, and loss of miR-122 is associated with metastasis and poor prognosis in hepatocellular carcinoma patients (7). We previously found that development of spontaneous hepatocellular carcinoma with age mimicked different stages of tumor progression (e.g., steatohepatitis, fibrosis, primary, and metastatic hepatocellular carcinoma) in miR-122 knockout (KO) mice (6, 9). We also observed that miR-122 depletion in the mouse liver leads to upregulation of chemokine (C-C motif) ligand 2 (CCL2), which recruits CCR2+CD11b+Gr1+ immune cells to the liver (8). These cells, in turn, produce proinflammatory cytokines, including IL6 and TNFα in the liver, resulting in hepatitis and eventually hepatocellular carcinoma.

CCL2 is known to be involved in the pathogenesis of several diseases characterized by mononuclear infiltrates, such as psoriasis, rheumatoid arthritis, and atherosclerosis (10, 11). CCL2 also promotes local inflammation and macrophage infiltration in the chronically injured liver (12). Very recently, Li and colleagues demonstrated that a chemical inhibitor of CCR2, the receptor of CCL2, inhibited hepatocellular carcinoma development by reducing monocytes/macrophage infiltration and M2 macrophage polarization as well as CD8+ T-cell...
activation in the murine xenograft model (13). However, whether targeting the CCL2–CCR2 axis by immunotherapy could be an effective therapeutic approach against chronic inflammation and hepatocellular carcinoma has not been addressed. In this study, we addressed this important question using a novel preclinical model, miR-122 KO mice that develop chronic inflammation–driven hepatocellular carcinoma (8). Our results clearly showed that targeting CCL2–CCR2 signaling by CCL2 immunotherapy could be an alternative approach in suppressing hepatitis and hepatocellular carcinoma development. Furthermore, our studies revealed that CCL2-neutralizing antibody (nAb) immunotherapy in miR-122 KO mouse model involves suppression of CD11b<sup>high</sup>Gr1<sup>+</sup> inflammatory myeloid cell recruitment and enhancement of natural killer (NK) cell cytotoxicity. These observations underscore the importance of targeting CCL2–CCR2 axis as a potential therapy in a subset of human hepatocellular carcinoma patients with chronic hepatitis inflammation and high CCL2.

### Materials and Methods

#### Treatment of miR-122 KO mice with CCL2 antibody and CCR2 inhibitor

miR-122 KO mice were generated as described previously (8). Animals were housed in a specific pathogen-free facility under a 12-hour light/dark cycle. Animals were handled following the guidelines of the Ohio State University Institutional Laboratory Animal Care Committee.

CCL2 nAb (anti-mouse CCL2; clone C1142) was generously provided by Janssen (14). miR-122 KO mice were injected intraperitoneally with CCL2 nAb (2 mg/kg). The control group was injected with vehicle (PBS). To study the function of CCL2 in hepatitis, 4-month-old KO mice were treated with CCL2 nAb twice a week for 4 weeks. To study the role of CCL2 in hepatocellular carcinoma development, 12-month-old KO mice were injected with CCL2 nAb twice a week for 8 weeks.

CCR2 inhibitor (Tocris Bioscience, cat# 2089) was given to KO mice in the drinking water daily (10 mg/kg) for 4 weeks. The control group for CCR2 inhibitor was fed with regular water.

#### Flow cytometric analysis

Murine mononuclear cells from livers were isolated as described previously (8). Erythrocytes were lysed using RBC lysis buffer (BioLegend). Cells isolated from livers were stained for immune cell surface markers for 30 minutes at 4°C. Following the function of CCL2 in hepatitis, 4-month-old KO mice were treated with CCL2 nAb twice a week for 4 weeks. To study the role of CCL2 in hepatocellular carcinoma development, 12-month-old KO mice were injected with CCL2 nAb twice a week for 8 weeks.

For staining of CD11b<sup>high</sup>Gr1<sup>+</sup>, NK1.1<sup>-</sup>, CD19<sup>-</sup>, CD69<sup>-</sup>, and IFN<sub>γ</sub> antibodies as described previously (8).

#### MRI

MRI of liver tumors in miR-122 KO mice was performed as described previously (17).

#### Serologic, histologic, and immunohistochemical analysis

Serum was isolated from mice by cardiac puncture after CO<sub>2</sub> asphyxiation and cervical dislocation and stored at −80°C. Biochemical analysis of serum enzymes was performed using VetACE (Alfa Wassermann system) as described previously (8). Tumor-derived NK cells were enriched by NK Cell Isolation Kit II (Miltenyi Biotec) and then sorted using a FACSAria II Cell Sorter (BD Biosciences). Hepa-1, a mouse hepatoma cell line, was generously provided by Dr. Gretchen Darlington (College of Medicine, Houston, TX). Although we did not authenticate these cells, they exhibited characteristics and gene expression profile of mouse hepatoma cells. Hepa cells were labeled with Chromium-51 (Cr-51) and then preincubated with C1142 antibody or isotype antibody at a concentration of 10 µg/mL for 1 hour. Then, a standard 4-hour Cr-51 release assay (16) was performed to access cytotoxicity of mouse NK cells against Hepa cells.

#### ELISA

For α-fetoprotein (AFP), mouse serum was collected by mandibular punch (before treatment) or cardiac punch (after treatment). KO mouse older than 10 months would be monitored for their body weight and serum AFP level by every 2 weeks. AFP level was quantified by DRG AFP (Alpha Fetoprotein) Kit (DRG, cat# EIA-1468).

For IFN<sub>γ</sub>, mouse liver mononuclear cells were isolated as described previously (8, 15). Hepa cells cultured in DMEM containing 10% FBS, were preincubated with C1142 (CCL2 nAb) at a concentration of 10 µg/mL for 1 hour. Then, 10<sup>6</sup> mononuclear cells were incubated with the same number of Hepa cells per well in a 96-well V-bottom plate at 37°C for 24 hours. Culture supernatants were collected for IFN<sub>γ</sub> estimation using mouse IFN<sub>γ</sub> ELISA Ready-SET-Go Kit (eBioscience, cat# 88-7314-88).

#### qRT-PCR

Total RNA was extracted from liver tissue using TRIzol (Life Technologies, cat#15596018) followed by DNase I treatment. DNase-treated RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat# 4368813). qRT-PCR analysis of each sample performed in triplicate was performed using SYBR Green chemistry. Gene expression was normalized to Gapdh and expressed as fold change of control.
expression was normalized to Gapdh. Relative expression was calculated by ΔΔCt method. Primer sequences are provided in the Supplementary Table S1.

Immunoblot analysis

Proteins were extracted from whole liver and tumor tissues by SDS lysis buffer, followed by immunoblotting with primary antibodies (8). Signals were developed using Pierce ECL reagent (ThermoFisher, cat# 32106) and quantified by ImageJ (imagej.nih.gov/ij/). Antibody information is provided in the Supplementary Material.

Statistical analysis

All bar diagrams are presented as mean ± SD. Two sample t tests or ANOVA were used for analysis for comparison of two or more groups. For the GEO data analysis, CCL2 expression from the quantile-normalized data was compared among tumor tissue types with ANOVA. Holm procedure was used to adjust for multiple comparisons. Fisher exact test was used to test the difference of tumor incidence and tumor size between the CCL2 nAb-treated and vehicle-treated groups. P values <0.05 were considered significant and represented as asterisks (*, P value ≤0.05; **, P value ≤0.01; ***, P value ≤0.001).

Results

CCL2 is upregulated in primary human hepatocellular carcinoma

To verify whether CCL2 plays a role in human hepatocellular carcinoma development, we first examined its expression across independent microarray datasets (hepatocellular carcinoma vs. normal liver) downloaded from Oncomine (18). To validate CCL2 expression, we chose The Cancer Genome Atlas, Mas liver, and Guichard liver that contain large patient cohorts (18–20). CCL2 DNA copy number or mRNA expression showed a significant increase in tumor tissues compared with the adjacent benign liver across four datasets (Table 1; refs. 18–20). To further evaluate CCL2 expression and disease progression, we analyzed hepatocellular carcinoma RNA microarray data (n = 115, mainly HCV positive) from GEO database (GSE14323; ref. 20). The results showed that CCL2 RNA expression was significantly elevated in hepatocellular carcinomas, cirrhotic livers, and cirrhotic hepatocellular carcinomas compared with normal livers (Fig. 1). These data imply that CCL2 might be critical for progression to cirrhosis and hepatocellular carcinoma. Thus, blocking the CCL2–CCR2 axis seems to be a reasonable therapeutic approach for hepatocellular carcinoma or even early stages of hepatocellular carcinoma development, such as hepatitis and cirrhosis.

CCL2–CCR2 blockade reduced chronic inflammation and liver injuries in miR-122 KO mice

CCL2 is a major chemokine that is known to cause various inflammatory diseases in humans (10, 21). Similarly, upregulation of CCL2 in miR-122 KO liver correlated with chronic inflammation (8). To determine whether CCL2 is a key player in hepatitis, and blocking CCL2 could inhibit liver inflammation, CCL2 nAb was administered to 4-month-old miR-122 KO mice by an intraperitoneal injection for 4 weeks (Fig. 2A). CCL2 nAb (C1142) was administered certain specificity toward murine CCL2 (14). In addition, CCL2 nAb was well tolerated in mice (14). Furthermore, its antitumor efficacy has been demonstrated in murine breast cancer metastasis (22), lung (23), brain (24), and prostate cancer (25, 26) models, and no adverse effects were reported.

CCL2 expression in human clinical samples (cancer vs. normal).

Table 1. CCL2 expression across four independent microarrays in hepatocellular carcinoma patients

<table>
<thead>
<tr>
<th>Cancer vs. normal</th>
<th>Array type</th>
<th>Fold change</th>
<th>Sample size</th>
</tr>
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<tbody>
<tr>
<td>Mas Liver</td>
<td>RNA</td>
<td>3.324</td>
<td>n = 115</td>
</tr>
<tr>
<td>Guichard Liver cohort 2</td>
<td>DNA</td>
<td>2.052</td>
<td>n = 52</td>
</tr>
<tr>
<td>TCGA Liver</td>
<td>DNA</td>
<td>2.063</td>
<td>n = 212</td>
</tr>
<tr>
<td>Guichard Liver cohort 1</td>
<td>DNA</td>
<td>2.153</td>
<td>n = 185</td>
</tr>
</tbody>
</table>

NOTE: CCL2 expression in human clinical samples (cancer vs. normal). Data provided by Oncomine (www.oncomine.com, January 2016, Thermo Fisher Scientific). TCGA database (TCGA Research Network: http://cancergenome.nih.gov) contains >200 hepatocellular carcinoma specimens of different etiology (HBV, HCV, alcohol, and NAFLD). Mas liver is an RNA array, which contains >100 hepatocellular carcinoma specimens (mostly HCV positive; ref. 20). The Guichard liver DNA array consisting of 237 hepatocellular carcinomas of various risk factors, including HBV, HCV, alcohol, and NAFLD, was analyzed by exosome sequencing and SNP array (19).

ALT and AST levels corroborated that the liver damage due to hepatitis in KO mice could be reversed by inhibition of CCL2 (Fig. 2D). Alkaline phosphatase (ALP), a marker of biliary dysfunction, was also reduced by approximately 50%; however, it was not statistically significant (Fig. 2D). This result implies that blocking CCL2 may not be able to fully rescue hepatobiliary damage because ALP is a target of miR-122, which is derepressed in miR-122–depleted livers (8, 9).

We have shown that activation of the CCL2–CCR2 axis in miR-122 KO liver correlates with the recruitment of CD11b<sup>high</sup>Gr1<sup>+</sup> cells and hepatic inflammation and injuries (8). In addition, CCR2 inhibitors showed anti-inflammatory effects in the animal models with different diseases, such as diabetic nephropathy (27), kidney hypertension (28), steatohepatitis (29), and renal atrophy (30) models, and no adverse effects were reported. We decided to test whether treatment of mice with a CCR2-specific inhibitor could reduce liver inflammation in KO mice. Indeed, KO mice fed water containing a CCR2 inhibitor exhibited reduced hepatic inflammation, as

Figure 2.
CCL2 nAb therapy reduces chronic liver inflammation and liver damage in adult miR-122 KO mice. A, The schematic presentation of the CCL2 nAb treatment. HCC, hepatocellular carcinoma. B, Liver histology of 4-month-old male KO mice injected intraperitoneally with CCL2 nAb (2 mg/kg, n = 5) or PBS (vehicle, n = 5). Representative images of H&E-stained liver sections (scale bars, 100 (top) and 25 µm (bottom)); Right, quantitation of the inflammation scores generated through blinded evaluation of H&E-stained liver sections (>100 magnification, 100 µm). The inflammatory area was quantified by ImageJ of three randomly chosen fields (>100 magnification, 100 µm) per animal (n = 5). C, Representative images of CD45-stained liver sections (scale bars, 100 (top) and 25 µm (bottom)); Right, CD45<sup>+</sup> areas quantified by ImageJ of three randomly chosen fields (>100 magnification, 100 µm) per animal (n = 5). D, Analysis of serum ALT, AST, and ALP levels (n = 5). n.s., not significant.
revealed by the dramatic reduction in bridging inflammation (Supplementary Fig. S2). Collectively, these data showed that blocking the CCL2–CCR2 axis could effectively inhibit hepatitis in miR-122 KO mice.

**CCL2 nAb therapy inhibited hepatitis by reducing CD11b<sup>high</sup>Gr1<sup>+</sup> inflammatory myeloid cell accumulation**

It is well established that immune cells play a critical role in liver inflammation (31). Our previous study showed that CD11b<sup>high</sup>Gr1<sup>+</sup> inflammatory myeloid cells excessively accumulated in miR-122-depleted livers (8). In addition, CD11b<sup>high</sup>Gr1<sup>+</sup> cells, which express CCR2 (8), are known to be recruited to the liver via CCL2–CCR2 interaction (32). Therefore, we quantitated CD11b<sup>high</sup>Gr1<sup>+</sup> cells in KO livers depleted of CCL2 or CCR2. Flow cytometric analysis of the immune cells isolated from livers and spleen showed reduced CD11b<sup>high</sup>Gr1<sup>+</sup> cell population in the KO mice treated with CCL2 nAb without significant change in their numbers in peripheral blood (Fig. 3A and B; Supplementary Fig. S3). Consistent with other studies (12, 13), we also found decreased number of hepatic macrophages in liver by IHC upon blocking the CCL2–CCR2 axis (Supplementary Fig. S4). However, no significant alterations in the number of hepatic B, T, and NK cells were observed (Supplementary Fig. S5A–S5D). In addition, immune cells isolated from CCR2 inhibitor–treated KO mice showed reduced hepatic CD11b<sup>high</sup>Gr1<sup>+</sup> cells (Supplementary Fig. S6), further supporting the idea that blocking the CCL2–CCR2 axis reduces CD11b<sup>high</sup>Gr1<sup>+</sup> inflammatory myeloid cells in liver.

IL6 and TNFα are two proinflammatory cytokines that drive hepatitis and hepatocellular carcinoma in the liver (33). Previously, we have shown that CD11b<sup>high</sup>Gr1<sup>+</sup> inflammatory myeloid cells produce IL6 and TNFα in the miR-122 KO liver (8). Therefore, it is expected that hepatic IL6 and TNFα levels...
would be suppressed if the population of CD11b<sup>+</sup>Gr1<sup>+</sup> cells is decreased in the CCL2 nAb-treated mice. Indeed, the expressions of both cytokines were reduced in the livers of CCL2 nAb-treated group (Fig. 3C). In addition, we also observed a decrease in Il1β expression upon CCL2 nAb treatment (Fig. 3C). Notably, CCL2 nAb treatment did not alter the expressions of other inflammatory cytokines or chemokines, such as Ccl5, Ccl8, Cxcl9, Il10, Ccl2, or Ccr2 (Fig. 3C). Taken together, these results suggest that inhibition of the CCL2–CCR2 axis reduces liver inflammation by suppressing CD11b<sup>+</sup>Gr1<sup>+</sup> inflammatory myeloid cells, which leads suppression of the two major proinflammatory cytokines, IL6 and TNFa.

Attenuation of hepatocarcinogenesis in miR-122 KO mice treated with CCL2 nAb

As aforementioned, CCL2 is highly expressed in the tumor tissues of hepatocellular carcinoma patients (Fig. 1; Table 1). Besides, blocking CCL2 suppresses chronic liver inflammation at early stages of hepatocellular carcinoma development in the KO mice (Fig. 2). Therefore, we speculated that impeding CCL2 function would suppress development of spontaneous liver tumors in KO mice. To test this, male KO mice (~12 months old) bearing liver tumors confirmed by the serum AFP levels were randomly assigned to two treatment groups.

Comparison of the serum AFP level with the tumor size in a large number of KO mice revealed that mice with serum AFP
levels >12 IU/mL usually developed tumors (Supplementary Fig. S7A). These ELISA data were further confirmed by MRI (Fig. 4A). KO mice were injected intraperitoneally with CCL2 nAb (2 mg/kg) or vehicle twice a week for 8 weeks (Fig. 4A), and the tumor burden was analyzed one week after the last injection. We chose male KO mice for this study as these mice, like men, have higher hepatocellular carcinoma penetrance and tumor burden (8, 9).

Significant phenotypic differences were observed in the liver between the vehicle- and the CCL2 nAb–treated groups after 8 weeks of CCL2 immunotherapy; however, there were no obvious changes in the appearance, activity, or body weight between the two groups (Supplementary Fig. S7B). Although the majority of the PBS-injected mice developed large macroscopic liver tumors, CCL2 nAb–treated mice mostly developed smaller and fewer tumors (Fig. 4B; Table 2). Consistent with the previous data (Fig. 3A), we found hepatic CD11b<sup>hi</sup>CD11c<sup>−</sup>Cr<sup>−</sup> cell population was reduced in the CCL2 nAb–treated tumor-bearing mice compared with vehicle-treated group (Fig. 4C). In addition, serum AFP and ALT levels were lower in the CCL2 nAb–treated mice (Table 2). Furthermore, the majority of the hepatocellular carcinomas in the vehicle-treated group were AFP and GPC3 positive and grade 2–3 (intermediate to poorly differentiated) hepatocellular carcinomas, whereas those in CCL2-inactivated mice were mostly grade 1 (well differentiated) hepatocellular carcinomas, AFP and GPC3 negative (Fig. 4D). These results suggest that CCL2 blockade is not only anti-inflammatory but also antitumorigenic in the liver.

Oncogenic signaling downstream of IL6 and TNFα was blocked in miR-122 KO tumors upon CCL2 nAb therapy

Because CCL2 nAb treatment suppressed the level of IL6 and TNFα in the liver (Fig. 3C), we hypothesized that the downstream oncogenic signaling pathways of IL6 and TNFα, namely STAT3 and NF-κB, respectively, would be inhibited in the CCL2-depleted mice. Indeed, immunoblot analyses showed that phospho-STAT3 (Y705) level was dramatically reduced, whereas total STAT3 level decreased only slightly in the tumors of CCL2 nAb–treated mice (Fig. 5A and C). Reduced nuclear STAT3 levels in the CCL2 nAb–treated mice confirmed that CCL2 inhibition suppresses STAT3 functions in liver tumors (Fig. 5B and C). Similarly, the level of two major NF-κB subunits, p65 and p50, was reduced in both whole liver lysates and nuclear extracts (Fig. 5B and C). The level of c-MYC, a downstream target of STAT3, was also reduced in the CCL2 nAb–treated mice (Fig. 5A and C). Histologically, p65 and c-MYC both showed reduced expression in the transformed hepatocytes, whereas pSTAT3 was decreased in immune cells as well as tumor cells (Supplementary Fig. S8).

Table 2. Histopathologic and serologic analysis of tumor-bearing miR-122 KO mice treated with CCL2 nAb for 8 weeks

<table>
<thead>
<tr>
<th>Vehicle/CCL2 nAb</th>
<th>HCC incidence</th>
<th>Tumor number</th>
<th>Sum of tumor diameter (cm)</th>
<th>Serum ALT (U/L)</th>
<th>Serum AFP (IU/mL)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>8/9</td>
<td>2.67 ± 122</td>
<td>1.71 ± 124</td>
<td>151.89 ± 68.06</td>
<td>14.07 ± 3.51</td>
<td>0.0004</td>
</tr>
<tr>
<td>CCL2 nAb</td>
<td>5/11</td>
<td>0.75 ± 0.79</td>
<td>0.57 ± 0.65</td>
<td>73.57 ± 28.5</td>
<td>9.95 ± 0.61</td>
<td>0.001</td>
</tr>
</tbody>
</table>

NOTE: All measurements are presented as mean ± SD. P values were calculated using Student two-tailed t test (for tumor number, sum of tumor diameter, tumor weight, ALT, and AFP) or Fisher exact test (for hepatocellular carcinoma incidence).

Abbreviation: HCC, hepatocellular carcinoma.

c-MYC is a well-known oncogene that promotes hepatocellular carcinoma proliferation (34, 35). Therefore, Ki-67 expression, a marker of cell proliferation, was evaluated in tumor cells by IHC. As expected, the number of Ki-67-positive cells was much less in both CCL2-depleted tumor and nontumor tissues (Fig. 5D; Supplementary Fig. S9). PCNA, another cellular proliferation marker, was also reduced in the CCL2 nAb–treated tumor extracts (Fig. 5E). Of note, we found cell apoptosis is increased in the CCL2 nAb–treated group as demonstrated by slight increase in cleaved PARP and cleaved caspase-7 levels (Supplementary Fig. S10). These data suggest that inhibition of hepatocellular carcinoma growth by targeting CCL2 is, at least in part, through the inhibition of STAT3 and NF-κB signaling and c-MYC expression.

CCL2 nAb activated NK cells in the tumor microenvironment to suppress liver cancer

Cancer immunotherapy has been widely conducted in the clinic over the past several years (36–38). As antibody-based immunotherapy is applied in this study, and NK cell is known to be activated by the Fc domain of antibodies (37), we investigated whether enhanced NK cytotoxicity could be a mechanism of tumor suppression in CCL2 nAb–treated mice. Although the total number of NK cells did not change significantly after CCL2 nAb immunotherapy (Supplementary Fig. S4C and S4D), the activity of NK cells increased, as demonstrated by the upregulation of hepatic IFNγ expression (Fig. 6A). To verify the source of the elevated IFNγ, primary hepatic immune cells isolated from tumor-bearing KO mice were cocultured with mouse hepatoma (Hepa1–6) cells in the presence of CCL2 nAb. The culture supernatants were subjected to IFNγ ELISA, and cells were analyzed by flow cytometry. Both ELISA and intracellular staining data showed that only NK cells (Fig. 6B and C) were the major producers of IFNγ. In addition, the cell surface expression of CD69, a marker for activated NK cells, increased when NK cells were cocultured with CCL2 nAb–treated mouse hepatoma (Hepa) cells (Fig. 6D), suggesting that NK cells could be activated upon exposure of hepatoma cells to CCL2 nAb. To confirm the activated NK cells could suppress cancer cell growth, NK-cell cytotoxicity was assessed by Cr-51 release assay (16, 39). To this end, Hepa cells were first labeled with Cr-51, followed by incubation with CCL2 nAb and subsequently, with tumor-derived NK cells. Enhanced release of Cr-51 from Hepa cells is indicative of higher cytotoxicity of NK cells. Indeed, tumor-derived NK cells cocultured with Hepa cells and CCL2 nAb exhibited higher cytotoxicity (Fig. 6E). This result also suggested that the increased cytotoxicity of NK cells could be triggered independent of other immune cells. Collectively, these results demonstrate that CCL2 nAb impedes tumor growth, at least in part, by activating NK cells.

Discussion

CCL2 is a chemotactic factor to tumor cells and inflammatory macrophage/monocytes. Blocking the CCL2–CCR2 axis by CCR2 inhibitor decreases the intrahepatic macrophage infiltration in a diet-induced steatohepatitis model (29). CCL2 Spiegelmer, a CCL2 RNA antagonist, suppresses the macrophage infiltration in the carbon tetrachloride (CCL4) and methionine–choline-deficient diet-induced liver injury models (12). Recently, Li and colleagues also demonstrated the importance of CCL2–CCR2 axis in different hepatocellular carcinoma models (13). In line
with the animal models mentioned above, it is clear that CCL2 is crucial for regulating the inflammatory milieu in liver. In this study, we aimed at elucidating the efficacy of CCL2 immunotherapy against chronic hepatitis and hepatocellular carcinoma in a murine model. This is a logical extension of our previous study that demonstrated a causal role of CCL2 in the inflamed liver that progressively leads to hepatocellular carcinoma in miR-122 KO mice (8). Analysis of GEO, Oncomine, and Protein Atlas databases showed increased CCL2 expression in patients suffering from hepatocellular carcinoma (18, 20, 40). These data suggest CCL2 may play a role in liver cancer. To further clarify the role of the CCL2–CCR2 axis in chronic liver inflammation and tumor development, we used CCL2 nAb to block CCL2 signaling. Our data suggest that CCL2 nAb inhibits tumor development in miR-122 KO mice through at least two distinct mechanisms: suppressing IL6 and TNFα production by reducing the infiltration of CD11b+Gr1+ inflammatory myeloid cells to the liver and enhancing NK-cell cytotoxicity (Supplementary Fig. S11).

An elegant study demonstrating the correlation of CCL2 protein level with poor survival of hepatocellular carcinoma patients was published (13) during the preparation of our manuscript. Li and colleagues showed that a clinically relevant CCR2 antagonist inhibited murine hepatocellular carcinoma progression by reducing M2-type tumor-associated macrophage population and

Figure 5. CCL2 nAb therapy suppresses hepatocellular carcinoma development in KO mice by inhibiting tumor cell proliferation. A, Immunoblotting of the key downstream proteins of IL6 and TNFα in KO tumors. B, Immunoblotting of NF-κB subunit and STAT3 in the tumor nuclear extracts. C, Representative images of Ki-67 staining (scale bar, 100 μm; inset, 25 μm). Quantitation was done by ImageJ. D, Representative images of PCNA staining (scale bar, 100 μm; inset, 25 μm). Quantitation was done by ImageJ of three randomly chosen fields (<200 magnification, 50 μm) per animal (n = 5). E, Immunoblotting of PCNA in liver tumors.
increasing CD8^+ cytotoxic T cells in orthotopic and subcutaneous hepatocellular carcinoma models (13). Their study supports the notion that blocking the CCL2–CCR2 axis has profound effects in modulating liver immune system to suppress hepatocellular carcinoma progression. While Li and colleagues found macrophage and CD8^+ T cells are regulated by a CCR2 inhibitor in xenograft models, we found CD11b^highGr1^+ inflammatory myeloid cells and NK cells could be modulated by CCL2 nAb in miR-122 KO mouse livers. Thus, these studies (ours and Li and colleagues’) demonstrated that targeting CCL2–CCR2 axis by blocking the receptor or the ligand could be an effective hepatocellular carcinoma therapy. In the present study, we used our unique animal model to study the role of CCL2 in liver inflammation and hepatocellular carcinoma progression in light of our previous work (8). We demonstrated two different types of immune cells, including CD11b^highGr1^+ inflammatory myeloid cells and NK cells, that play key roles in regulating liver inflammation and tumor development after blocking CCL2 function. In addition, CD11b^highGr1^+ inflammatory myeloid cells promote tumorigenesis by increasing tumor cell proliferation via IL6 and TNFα. Both IL6 and TNFα are well-known proinflammatory cytokines that activate oncogenic transcription factor STAT3 to promote hepatocellular carcinoma development (33, 41). Consistent with the previous findings, our

Figure 6.
current data reveal that CCL2 nAb reduces accumulation of CD11b<sup>+</sup>Gr1<sup>+</sup> cells, which decreases IL6 and TNFα levels in the tumor and surrounding liver tissues, and reduces the activation of STAT3 and the expression of c-MYC. Ours and Li and colleagues’ data demonstrated that targeting the CCL2–CCR2 axis by blocking the receptor or the ligand could be an effective hepatocellular carcinoma therapy. In future, it would be interesting to investigate whether the combination of both would be a more effective therapeutic approach.

NK cells are key modulators of liver diseases. Activated NK cells suppress liver disease progression from chronic hepatitis to hepatocellular carcinoma (42). On the basis of ours and Li and colleagues’ data (13), CCL2 is expressed in liver tumors of both mouse and human origin. The CCL2 nAb binds to Fc receptor (CD16) on NK cells (37), and the CCL2 secreted from tumor cells binds to the CCL2 nAb. This can trigger NK-cell activation (i.e., enhancement of IFNγ secretion and cytotoxicity), causing enhanced killing of liver tumor cells. In this study, we observed enhanced cytotoxic capability of NK cells against CCL2 nAb–treated hepatoma cells. We also noticed that the level of IFNγ was higher in the NK cells isolated from the livers of tumor-bearing KO mice treated with CCL2 nAb, reinforcing the notion that NK cells were activated by the CCL2 nAb. Consistent with other literatures that cytotoxic NK cells activate caspase-3, -7, and PARP (43, 44), we found increased cleaved caspase-7 and cleaved PARP in the CCL2 nAb–treated mice (Supplementary Fig. S10). Inhibition of STAT3 activity has been shown to sensitize hepatocellular carcinoma cells to NK cell–mediated cytotoxicity (45). Interestingly, our data show that STAT3 activity is reduced in the CCL2 nAb–treated mice (Fig. SA–C), suggesting a reciprocal regulation.

Novel therapeutic strategies for hepatocellular carcinoma are urgently needed, as existing therapies could not cure or prolong patients’ survival even by 6 months (5). In this study, we highlight the importance of CCL2 in contributing to chronic liver inflammation and tumorigenesis. Furthermore, the efficacy of a CCL2–specific nAb in the miR-122 mouse model underscores the feasibility of immune-based therapy in inflammatory liver disease and cancer. Our study shows that CCL2 nAb immunotherapy elicits two distinct mechanisms that act in concert to inhibit hepatitis and hepatocellular carcinoma development, and provides rationale for future clinical trials in hepatocellular carcinoma patients with chronic inflammation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conceptualization and design: K.-Y. Teng, L.A. Snyder, J. Yu, K. Ghoshal

Development of methodology: K.-Y. Teng, J. Han, S. He, K. Ghoshal

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.-Y. Teng, J. Han, S.-H. Hsu, S. He, J. Barajas, K. Ghoshal

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.-Y. Teng, J. Han, X. Zhang, J. Yu, K. Ghoshal

Writing, review, and/or revision of the manuscript: K.-Y. Teng, J. Han, X. Zhang, S.-H. Hsu, L.A. Snyder, W.L. Frankel, M.A. Caligiuri, J. Yu, K. Ghoshal

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**References**

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