Systemic Agonistic Anti-CD40 Treatment of Tumor-Bearing Mice Modulates Hepatic Myeloid-Suppressive Cells and Causes Immune-Mediated Liver Damage

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Abstract

Immune-stimulatory mAbs are currently being evaluated as antitumor agents. Although overall toxicity from these agents appears to be moderate, liver toxicities have been reported and are not completely understood. We studied the effect of systemic CD40 antibody treatment on myeloid cells in the spleen and liver. Naïve and tumor-bearing mice were treated systemically with agonistic anti-CD40 antibody. Immune cell subsets in the liver and spleen, serum transaminases, and liver histologies were analyzed after antibody administration. Nox2⁺/−, Cd40⁺/−, and bone marrow chimeric mice were used to study the mechanism by which agonistic anti-CD40 mediates its effects in vivo. Suppressor function of murine and human tumor-induced myeloid-derived suppressor cells (MDSC) was studied upon CD40 ligation. Agonistic CD40 antibody caused liver damage within 24 hours after injection in two unrelated tumor models and mice strains. Using bone marrow chimeras, we demonstrate that CD40 antibody–agonistic CD40 antibodies, leading to tumor rejection in animal models.

Introduction

The TNF receptor family member CD40 is a stimulatory molecule constitutively expressed on a large variety of cells, including dendritic cells, B cells, macrophages, and endothelial cells (1–5). CD40 engagement of antigen-presenting cells provides the “license” to T-cell help and enhances T-cell activation (6, 7). Agonistic CD40 antibodies were shown to overcome T-cell tolerance in tumor-bearing mice and facilitate development of potent cytotoxic T-cell responses by enhancing the effects of cancer vaccines (8–12). Recently, immune-modulatory regimens—cytokine therapy (1–5, 13–17), radiotherapy (6, 7, 18–20), chemotherapy (8–12, 21–23), kinase inhibitors (24), or monoclonal antibodies (25)—have been shown to synergize with agonistic CD40 antibodies, leading to tumor rejection in animal models.

However, systemic administration of immunostimulatory CD40 antibodies has been associated with cytokine release syndrome, lymphopenia, and liver toxicity in clinical trials (1, 3–5). In preclinical models, Fransen and colleagues (6) observed that intravenous delivery of high- or low-dose agonistic CD40 antibody increased liver toxicity in mice bearing virally transformed tumors. Agonistic anti-CD40 biodistribution experiments by Sandin and colleagues (9) showed that systemic administration led to higher antibody concentrations in the liver compared with local delivery. However, the reason why systemic agonistic CD40 antibody causes liver toxicity remained unknown.

Tumor-induced myeloid-derived suppressor cells (MDSC) constitute one of the main players in tumor-induced immune suppression. They comprise a heterogeneous population of myeloid cells of diverse differentiation status, whose main feature is the suppression of innate and adaptive immune responses (8). Our laboratory and others have previously described that tumor-induced CD11b⁺Gr-1⁺ MDSC accumulate in the liver of mice (13, 15, 17) and in patients with hepatocellular carcinoma (18, 20). In addition, hepatic MDSC have been reported to promote the generation of liver metastasis (21), and CD11b⁺CCR2⁺ cells have been detected in liver metastasis from patients with colorectal cancer (26). However, little is known about the biology of tumor-induced hepatic MDSC.
In the study reported herein, we investigated the effect of agonistic anti-CD40 antibody injection on hepatic and splenic MDSC in tumor-bearing mice. Although agonistic anti-CD40 treatment led to severe, MDSC-mediated hepatitis in mice, we also provide evidence suggesting that MDSC mature into a proinflammatory cell type with less arginase activity. These results are recapitulated in human CD14+ HLA-DRlow MDSC, which also lose arginase expression and thereby suppressor function in vitro.

Materials and Methods

Mice and cell lines

Eight- to 10-week-old female BALB/c, C57BL/6–CD45.2, and BL6–CD45.1 were purchased from NCI/Frederick. H-2Kb OVA57-264 TCR transgenic OT-I Cd40−/− (purchased from The Jackson Laboratories), and Nox2−/− mice (a kind gift from Robert Mumford, NCI) were bred at NCI/Frederick. Bone marrow chimeric mice were generated as previously described (27). Bone marrow chimerism was confirmed 4 weeks after bone marrow transplant and was above 80%. EL4 and B16 GM-CSF cells were a kind gift of Dr. Drew Pardoll (The Johns Hopkins University, Baltimore, MD) and previously used (27). 4T1 cells were kindly provided by Christopher A. Klebanoff (NCI). RIL-175 hepatocellular carcinoma cell line was obtained from Dr. Lars Zender (University Hospital of Tubingen, Germany) and used recently (13, 28). All tumor cell lines used were tested negative for mycoplasma using a MycoAlert Plus kit (Lonza) routinely. Last test was performed on December 2014. Mice were injected subcutaneously in the flank with 1 × 10⁵ tumor cells. Tumor size was measured twice a week. Metastatic tumors were established in the liver by intrasplenic injection of 3 × 10⁵ EL4 cells (29). Mice received antibody treatment 3 weeks after tumor cell inoculation into the spleen. All mice were handled, fed, and housed in accordance with the U.S. Department of Health and Human Services institutional guidelines.

In vivo antibody treatment

Tumor-free littermates or mice bearing subcutaneous tumors between 10 and 15 mm maximum diameter were inoculated i.p. with 100 µg of rat anti-mouse agonist CD40 antibody (clone FK-45; BioXCell) or irrelevant rat IgG2a (2A3; BioXCell). Mice were sacrificed 24 hours after injection. Alanine/aspartate aminotransferase (ALT/AST) levels were determined in mouse sera by biochemistry analysis in the Department of Laboratory Medicine (NCI). Serum TNFα levels were quantified by ELISA following the manufacturer’s instructions (eBioscience). Arginase activity in cell lysates was determined as described (31). For ovalbumin (OVA) cross-presentation, 1 × 10⁵ CD11b+ cells were cultured for 24 hours alone or in the presence of 0.1 µg of rat anti-mouse CD40 antibody. Cells were washed twice with PBS, and OT-I CD8+ T cells were MACS-sorted using a mouse CD8+ T cell isolation kit (Miltenyi Biotec), added to the culture in a 1:1 ratio, and stimulated with 0.1 µg/mL OVA-derived SIINFEKL peptide overnight. IFNγ production by OT-I CD8+ T cells was determined by intracellular staining.

Determination of hepatocyte cytotoxicity by hepatic CD11b+ cells

Luciferase-expressing RIL-175 hepatoma target cells were cultured at a 1:50 (target: effector) ratio with EL4-induced hepatic CD11b+ cells isolated from mice 3 hours after treatment with 100 µg of either IgG or anti-mouse CD40. After 16 hours, the number of surviving adherent cells was evaluated using Dual Luciferase Reporter Assay (Promega). H2O2 (2 mmol/l, Invitrogen) and 100 U/ml catalase (Sigma) were used for apoptosis induction and blocking of ROS release, respectively.

Adaptive cell transfer

Hepatic CD45.1+CD11b+ cells were MACS-isolated from B16 GM-CSF tumor-bearing mice, because GM-CSF-expressing tumors have been shown to support the accumulation of large numbers of CD11b+Gr-1− cells in spleen and liver (13). CD11b+ cells (5 × 10⁵) were injected into the tail vein of tumor-free CD45.2+CD40−/− mice. In another set of experiments, 5 × 10⁷ CD11b+ cells from B16 GM-CSF tumor-bearing wild-type (WT) or Nox2−/− mice were injected into the tail vein of tumor-free CD45.2+CD40−/− mice. Mice were subsequently inoculated i.p. either with 100 µg of anti-mouse CD40 or isotype control. Mice were sacrificed 16 hours after antibody injection.

Human MDSC studies

Peripheral blood mononuclear cells (PBMC) were obtained from NIH Blood Bank (healthy donors) and patients with gastrointestinal-related cancer (see Supplementary Information). Written consent was obtained from all patients before blood sampling on a research protocol approved by the NCI Institutional Review Board. FACS-sorted CD14+ HLA-DRhigh and CD14+ HLA-DRlow cells were purified as previously described (32). When indicated, 1 × 10⁶ or 2 × 10⁶ sorted cells were cultured in complete RPMI medium with or without 0.1 µg/mL megaCD40L (Enzo Life Sciences) or 5 µg/mL anti-human CD40 antibody (clone 82111; R&D systems) for 24 hours. Then, total RNA was

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isolated using an RNeasy kit (Qiagen). cDNA synthesis was carried out using an iScript CDNA synthesis kit according to the manufacturer’s instructions (Biorad). Arginase-1 qPCR (primer sequences available upon request) was performed using IQ SYBR Green Supermix and thermal cycler (Applied Biosystems). Triplicate reactions were performed for each sample, and expression of tested gene was normalized relative to levels of GAPDH. To assess human MDSC function, CD14+/HLA-DRlow cells were isolated from healthy donor PBMC and incubated at different ratios with CD3/CD28 (Miltenyi Biotec)-stimulated CD8+ T cells previously isolated from the remaining CD14− fraction (human CD8+ T cell isolation kit; Miltenyi Biotec) in the presence or absence of 0.1 μg/mL megaCD40L. Proliferation was measured 72 hours later by incorporation of 3H Thymidine (Amersham).

Statistical analysis
Analyses were performed with GraphPad Prism software. Data are provided as mean ± SEM, unless indicated otherwise. For comparisons between two groups, statistical analyses were performed using a Student t test, and P < 0.05 was considered significant. For comparisons involving three groups or more, a one-way ANOVA using the Kruskal–Wallis test was performed (P < 0.05) was considered statistically significant. When analyzing the response of two populations to two or more treatments, two-way ANOVA was performed (P < 0.05) was considered statistically significant. Symbols indicating statistical significance are as follows (unless indicated otherwise): n.s., not statistically significant; *, P < 0.05; ***, P < 0.01; ****, P < 0.005.

Results
Systemic agonistic anti-CD40 induces immune-mediated acute hepatitis in tumor-bearing mice
We treated naïve tumor-free and EL4 tumor-bearing mice with agonistic anti-CD40 or isotype IgG control. Systemic CD40 antibody treatment resulted in a significant increase of serum ALT and AST levels in tumor-bearing mice (ALT, 5,529 ± 647 U/L; AST, 6,687 ± 1,166 U/L) compared with tumor-free mice (ALT, 528 ± 170 U/L; AST, 973 ± 302 U/L) and with tumor-bearing mice treated with IgG (ALT, 122 ± 21 U/L; AST, 465 ± 108 U/L, Fig. 1A). Similarly, in BALB/c mice (Fig. 1B), transaminases were elevated in EL4 tumor-bearing mice 24 hours after treatment compared with tumor-free mice and with tumor-bearing mice treated with control IgG (Fig. 1C). In summary, ROS release plays a role in systemic CD40 agonist-mediated hepatitis (data not shown). Histologic studies revealed milder immune cell infiltrate, but still evidence of endothelial inflammation and injury in agonistic CD40 antibody–treated Nox2−/− mice (Fig. 2B and C). Although the pattern of liver injury was similar in WT and Nox2−/− tumor-bearing mice, Nox2−/− mice showed less confluent parenchymal necrosis involving only 2% to 3% of the cross-sectional area. Furthermore, fewer fibrin thrombi were observed in the outflow veins. In line with this finding, systemic CD40 agonist treatment resulted in a significant increase of ROS production by hepatic CD11b+ Gr-1+ cells in tumor-bearing mice compared with tumor-free mice and with tumor-bearing mice treated with control IgG (Fig. 2D). To find out the contribution of hepatic myeloid cells in hepatocyte cell death, we isolated hepatic CD11b+ cells from tumor-bearing mice 3 hours after treatment either with agonistic anti-CD40 or isotype control and cocultured them with luciferase-expressing hepatoma cells. Using luciferase expression as readout of cell viability, luciferase signal was decreased when hepatoma cells were incubated with hepatic CD11b+ cells from agonistic anti-CD40-treated mice (Fig. 2E). CD11b−-mediated cell death was blocked in the presence of the ROS inhibitor catalase, suggesting that CD40 ligation exacerbates ROS-mediated liver cell killing by tumor-induced hepatic myeloid cells. In summary, ROS release plays a role in systemic CD40 agonist–mediated hepatitis.

Agnostic CD40 antibody modulates tumor-induced hepatic CD11b+ Gr-1+ cells
Next, we studied CD11b+ Gr-1+ cells after agonistic anti-CD40 treatment. We found that the absolute cell number of hepatic CD11b+ Gr-1+ cells increased in EL4 tumor-bearing mice 24 hours after agonistic anti-CD40 injection (tumor-free 4.4 × 105 ± 1.4 × 105 vs. tumor-bearing 1.1 × 106 ± 1.9 × 106, P = 0.052; Fig. 3A).
This increase was significantly higher in the CD11b+Gr-1low monocytic cell subset (M-MDSC) than in the CD11b+Gr-1high granulocytic subset (G-MDSC; Supplementary Fig. S3A and S3B).

Apart from CD11b+Gr-1+ cell changes, a moderate decrease of CD3+CD19+ B cells and an increase of CD11c+ dendritic cells were observed upon agonistic CD40 antibody injection.
Interestingly, agonistic anti-CD40 treatment significantly increased the absolute number of CD11b+ and CD80-expressing CD11b+Gr-1+ cells (Supplementary Fig. S4 and Fig. 3B and C). Similar results were observed in 4T1 tumor-bearing mice (Supplementary Fig. S3I and S3J). To address whether the effects of agonistic anti-CD40 were restricted to hepatic myeloid cells or whether this was a systemic effect, we also studied splenic CD11b+Gr-1+ cells in our system. An increase in the absolute numbers of CD11b+Gr-1+ cells and more CD40- and CD80-expressing CD11b+Gr-1+ cells was also seen in spleens of tumor-bearing mice treated with agonistic anti-CD40 (Fig. 3D–F). We further analyzed MDSC subsets and found that both hepatic G-MDSC and M-MDSC accumulated over time after anti-CD40 agonist injection (Supplementary Fig. S5A and S5B). However, hepatic G-MDSC produced more ROS than M-MDSC in response to CD40 ligation in vivo (Supplementary Fig. S5C).

Finally, we studied the effects of agonistic anti-CD40 on hepatic CD11b+ cells in vitro. Here, a significant decrease in their arginase activity was seen upon anti-CD40 treatment (Fig. 3G). Moreover, anti-CD40 treatment improved their ability to induce antigen-specific IFNg release by CD8+ T cells (Fig. 3H). Finally, significant levels of TNFa were detected in cell supernatants after incubation of tumor-induced hepatic myeloid cells with CD40 antibody (Fig. 3I). In summary, CD40 ligation on tumor-induced hepatic myeloid cells results in enhanced maturation and activation in vivo and in vitro.

Tumor-induced hepatic CD11b+ cells mediate liver inflammation upon systemic agonistic CD40 antibody

To provide a direct link between the presence of hepatic myeloid cells and anti-CD40-mediated liver inflammation, we transferred WT tumor-induced hepatic CD45.1+CD11b+ cells,
where 80% were CD11b⁺Gr-1⁺ MDSC (Supplementary Fig. S6A and S6B), into CD45.2⁺Cd40⁻/Cd80⁻/Cd86⁻/Cd16⁻ recipients followed by systemic injection of the agonistic anti-CD40. Although agonistic CD40 antibody did not cause inflammation to regular Cd40⁻/Cd80⁻/Cd86⁻/Cd16⁻ mice, transfer of tumor-induced hepatic CD45.1⁺myeloid cells and subsequent agonistic anti-CD40 injection to the Cd40 knockouts resulted in ALT and AST serum elevation (Fig. 4A and B). In addition, an increase in TNFα serum levels was observed (Fig. 4C). Consequently, CD40 ligation on tumor-induced hepatic myeloid cells resulted in enhanced inflammation. To address the role of myeloid-derived ROS in this setting, we transferred either WT or Nox2⁻/⁻ tumor-induced hepatic CD11b⁺ cells into Cd40⁻/⁻ recipients and then challenged them with agonistic anti-CD40. Transfer of WT tumor-induced hepatic myeloid cells and subsequent agonistic anti-CD40 injection to the Cd40 knockouts resulted in higher ALT serum levels compared with Nox2⁻/⁻ tumor-induced hepatic CD11b⁺ cell transfer (Fig. 4C). CD40 ligation impairs immunosuppressive function of human CD14⁺HLA-DRlow MDSC

We isolated human CD14⁺HLA-DRlow MDSC from PBMC of healthy controls (Fig. 5A) or patients (Fig. 5B) with gastrointestinal cancer (Supplementary Table S1). CD40 engagement using multivalent CD40L reduced arginase-1 mRNA expression in CD40⁻/⁻ recipient mice. A-C. absolute number of hepatic CD11b⁺Gr-1⁺ cells (A) expressing CD40 (B) and CD80 (C) 24 hours after injection. D-F. absolute number of splenic CD11b⁺Gr-1⁺ cells (D) expressing CD40 (E) and CD80 (F). A-F. data expressed as mean ± SEM are representative of three independent experiments. EL4-induced liver CD11b⁺ cells (1 x 10⁶; n = 3/mice) were cultured in the presence or absence of 0.1 µg CD40 Ab. Arginase activity (G) determined by urea release in the cell lysates was measured with a colorimetric assay 16 hours after incubation. H. EL4-induced liver CD11b⁺ cells (1 x 10⁶; n = 3/mice) were cultured in the presence or absence of 0.1 µg CD40 Ab. After 24 hours, cells were washed with PBS and incubated overnight with 1 x 10⁵ sorted CD8⁺OT-I OVA-specific T cells in the presence of 0.1 µg/mL OVA. Intracellular IFNγ production by CD8⁺ T cells is shown. Data shown as mean ± SEM are representative of two independent experiments. EL4-induced liver CD11b⁺ cells (1 x 10⁶; n = 3/mice) were cultured in the presence or absence of 0.1 µg CD40 Ab. TNFα from the cell culture supernatants was determined by ELISA. Data shown as mean ± SEM are representative of two independent experiments. n.s., not statistically significant; *, P < 0.05; **, P < 0.01; ***, P < 0.005; (A–G) Student t test, (H) one-way ANOVA.
Agonistic Anti-CD40 Activates Tumor-Induced CD11b\(^+\)Gr-1\(^+\) Cells

Figure 4. Tumor-induced myeloid cells enhance liver inflammation in vivo upon systemic agonistic anti-CD40 treatment. B16 GM-CSF-induced WT liver CD45\(^{+}\)CD11b\(^+\)Gr-1\(^+\) cells (5 \times 10\(^5\); \(n = 3\) mice) were injected i.v. into tumor-free (TF) CD45.2\(^+\)CD40\(^–\) mice (\(n = 6-8\) mice/group). Then either CD40 Ab or IgG was injected i.p. either CD40 Ab or IgG was injected i.p. mouse received CD40 Ab as control (\(n = 3\) mice/group). Serum ALT and AST (A) levels as well as serum TNF\(_\alpha\) (B) were measured 16 hours after injection. Cumulative data shown as mean \(\pm\) SEM are representative of two independent experiments. B, data shown as mean \(\pm\) SEM are representative of two independent experiments. C, 5 \times 10\(^5\) B16 GM-CSF-induced WT or Nox2\(^–/–\) liver CD11b\(^+\) cells (\(n = 2\) mice/group) were injected i.v. into tumor-free CD45.2\(^+\)CD40\(^–\) mice (\(n = 6-7\) mice/group). Then either CD40 Ab or IgG was injected i.p. Tumor-free CD40\(^–\) mice received CD40 Ab as control (\(n = 2\) mice). Serum ALT levels were measured 16 hours after injection. Cumulative data shown as mean \(\pm\) SEM are representative of two independent experiments. n.s., not statistically significant; *, \(P < 0.05\); one-way ANOVA.

Discussion

The approval of ipilimumab by the FDA in 2011, and very recently pembrolizumab and nivolumab, has sparked great interest in immune checkpoint inhibitors in oncology in recent years (36). Similarly, agonistic antibodies to TNF receptor molecules, such as activating CD137, OX40, and CD40 antibodies, have shown promising results in both preclinical and early clinical settings (5, 37, 38). Different mechanisms of action have been described for agonistic anti-CD40 antibody therapy in cancer (37). Here, we studied the effect of systemic anti-CD40 treatment in tumor-bearing mice on hepatic and splenic MDSC. We found that agonistic CD40 antibody triggered immune-mediated and ROS-dependent acute liver damage in tumor-bearing mice by activating hepatic CD11b\(^+\)Gr-1\(^+\) cells. Further studies provide preliminary evidence suggesting that agonistic anti-CD40 treatment causes maturation and loss of suppressor function of hepatic and systemic murine as well as human MDSC.

Various cells in the liver, including hepatocytes, Kupffer cells, and different myeloid cells, express CD40 (39). Previously, transaminases had been reported in patients treated with agonistic anti-CD40 in a phase I trial (1). Similarly, liver toxicity upon agonistic anti-CD40 treatment has been observed in preclinical models upon intravenous administration, but this was reduced when the antibody was injected peritumorally (6). This adverse event was initially attributed to a direct effect of agonistic anti-CD40 on CD40-expressing hepatocytes (33). Our findings suggest a potential alternative explanation, namely the engagement of hepatic CD11b\(^+\)Gr-1\(^+\) MDSC and ultimately ROS release leading to hepatocyte death upon anti-CD40 treatment. There is a preferential accumulation of MDSC in the liver of tumor-bearing mice, which are in larger numbers than other myeloid populations such as Kupffer cells (13, 15, 17, 28). These cells express low levels of CD40 (40–42), which can be further enhanced upon incubation with IFN\(\gamma\) (43) as well as in a setting of acute inflammation (data not shown). Our data using bone marrow chimeric mice clearly suggest a pivotal role for CD40-expressing myeloid cells in CD40-mediated liver damage in tumor-bearing mice. First, serum transaminases and TNF\(_\alpha\) serum levels were higher in CD40\(^–/–\) mice.
reconstituted with WT bone marrow than in WT mice after reconstitution with CD40−/− bone marrow. Second, adoptive transfer of WT MDSC into CD40−/− mice followed by agonistic anti-CD40 treatment resulted in increased transaminases and TNFα serum levels.

Oxidative stress via ROS release by mononuclear phagocytes plays a pivotal role in inflammatory liver injury (35). Treatment of mice with free radical scavengers decreased ALT in a model of immune-mediated hepatitis (44). Using phagocytic knockouts, we observed that knockout tumor-bearing mice had lower ALT/AST levels than tumor-bearing littermate controls, suggesting a ROS-mediated liver cell damage in our model. We provide further biologic evidence by showing that tumor-induced hepatic myeloid cells produce ROS upon CD40 ligation, which in turn induces hepatocyte cell death.

Our data show that systemic administration of agonistic anti-CD40 increased the accumulation of CD11b+Gr-1− cells in the liver of tumor-bearing mice. Our experiments did not address a possible role for liver-infiltrating neutrophils, which accumulate in murine models of immune-mediated hepatitis by margination of neutrophils through sinusoids (45). Inflammatory neutrophils express CD11b and high levels of Gr-1 similar to the granulocytic subset of tumor-induced hepatic MDSC. However, we provide multiple lines of evidence suggesting that tumor-induced hepatic CD11b+Gr-1− cells and not inflammatory neutrophils were responsible for agonistic anti-CD40-mediated liver toxicity: (i) Transaminase levels were higher in tumor-bearing mice (in which hepatic MDSC accumulate) than in naive mice after agonistic anti-CD40 treatment; (ii) significant accumulation of CD11b+Gr-1− mononuclear-like MDSC rather than CD11b+Gr-1− granulocytic-like MDSC was observed in tumor-bearing mice upon agonistic CD40 antibody–driven liver toxicity; and (iii) transfer of tumor-induced hepatic myeloid cells into CD40−/− mice caused ALT/AST elevation upon agonistic anti-CD40. In this experiment, only tumor-induced MDSC expressed CD40.

Our data suggest that tumor-induced hepatic and— to a lesser extent— splenic CD11b+Gr-1− cells increase CD40 and CD80 surface marker expression upon agonist CD40 antibody treatment. This study, along with others, shows that tumor-induced CD11b+Gr-1− MDSC express low levels of CD40 (2, 46) and CD80 (7, 47). Because CD40 ligation induces upregulation of CD80 (10–12, 48), our results suggest that hepatic tumor-induced CD11b+Gr-1− cells may mature and get activated by CD40 ligation in vivo. However, in the absence of specific markers to clearly separate tumor-induced myeloid cells with suppressor function from other innate immune cells, which migrate to the liver in acute inflammatory settings (14, 16, 45), we could not formally prove the plasticity of hepatic MDSC.

CD14+HLA-DRlow MDSC accumulate in the peripheral blood and tumors from patients with different types of cancer, including liver cancer (18–20, 49). Although an accumulation of CD11b+CCR2+ cells has been described in sections obtained from patients with colorectal liver metastasis (22, 23, 26), it is not known so far whether MDSC also accumulate in tumor-free livers of patients with cancer. Therefore, at this point, one can only speculate whether hepatic MDSC caused ALT/AST elevations in patients.
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