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

José Medina-Echeverrz, Chi Ma, Austin G. Duffy, Tobias Eggert, Nga Hawk, David E. Kleiner, Firouzeh Korangy, and Tim F. Greten

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-bearer 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. Noc2+/−, 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 have been 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 proinflammatory cell type with less arginase activity. These results are recapitulated in human CD14\(^+\)HLA-DR\(^{low}\) 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, and C57BL/6.CD45.1 were purchased from NCI/Frederick. H-2K\(^b\) OVA\(^{257-264}\) TCR transgenic OT-I, BD6-CD45.1 were bred at NCI/Frederick. 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 Tübingen, 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 \(1 \times 10^{5}\) tumor cells. Tumor size was measured twice a week. Metastatic tumors were established in the liver by intrasplenic injection of \(3 \times 10^{5}\) 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 F9K-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). Hematocruin–eosin-stained liver tissues were analyzed by a pathologist (D.E. Kleiner) in a blinded fashion.

**Flow cytometry analysis**

Liver mononuclear cells were isolated from tumor-bearing C57BL/6 mice before and after injection of 0.1 mg of either isotype or anti-mouse CD40 antibody. Supernatants were collected and TNFα production by OT-I CD8\(^+\) T cells was determined (Milenyi 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.

**Functional assays in vitro**

Reactive oxygen species (ROS) production was determined using Carboxy-H2DCFDA (Invitrogen) as described by Corzo and colleagues (30). OVA257-264 TCR transgenic OT-I, BD6-CD45.1 were 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 Tübingen, 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 \(1 \times 10^{5}\) tumor cells. Tumor size was measured twice a week. Metastatic tumors were established in the liver by intrasplenic injection of \(3 \times 10^{5}\) 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 FGK-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). Hematocruin–eosin-stained liver tissues were analyzed by a pathologist (D.E. Kleiner) in a blinded fashion.

**Flow cytometry analysis**

Liver mononuclear cells were isolated from tumor-bearing mice before and after injection of 0.1 mg of either isotype or anti-mouse CD40 antibody. Supernatants were collected and TNFα production by OT-I CD8\(^+\) T cells was determined (Milenyi 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.

**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-DR\(^{high}\) and CD14\(^+\)HLA-DR\(^{low}\) cells were cultured as previously described (32). When indicated, \(1 \times 10^5\) or \(2 \times 10^5\) 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...
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 the tested gene was normalized relative to levels of GAPDH. To assess cell viability, luciferase signal was measured using a luciferase assay kit. Statistical analysis was 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 or more groups, a one-way ANOVA 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 < 0.005.

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 in serum ALT and AST levels in tumor-bearing mice (ALT, 5,529 ± 647 U/L; AST, 2,667 ± 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 4T1 tumor-bearing mice treated with agonistic anti-CD40 (ALT, 170 ± 20 U/L; AST, 415 ± 62 U/L) compared with tumor-bearing mice treated with isotype control (ALT, 57 ± 11 U/L; AST, 220 ± 21 U/L). Time course studies demonstrated a peak for ALT and AST serum levels after 24 hours in EL4 tumor-bearing mice and a return to baseline within 72 hours (Supplementary Fig. S1A and S1B). Histologic analysis of liver sections from EL4 tumor-bearing mice revealed signs of severe hepatitis with inflammatory cell infiltration and confluent parenchymal necrosis (Fig. 1C and D). Similar results were obtained in 4T1 tumor-bearing BALB/c littermates (Supplementary Fig. S1B and S1C). Furthermore, in a model of EL4-induced liver metastases (Supplementary Fig. S2A), systemic anti-CD40 agonist increased serum ALT and AST levels (ALT, 2,096 ± 759 U/L; AST, 5,645 ± 1,320 U/L) compared with IgG-treated mice (ALT, 414 ± 92 U/L; AST, 2,542 ± 619 U/L; Supplementary Fig. S2B).

CD40 is not only expressed by liver-infiltrating immune cells but also by parenchymal cells such as hepatocytes (33). Therefore, we generated Cd40 knockdown bone marrow chimeras to dissociate the relative contribution of the liver immune cell compartment to CD40 antibody–mediated liver damage from direct ligation of CD40 antibody on CD40-expressing hepatocytes. Transaminases were clearly elevated in EL4 tumor-bearing Cd40−/− mice reconstituted with WT bone marrow upon CD40 ligation (ALT, 437 ± 62 U/L; AST, 1,207 ± 230 U/L; Fig. 1E). In contrast, tumor-bearing WT mice reconstituted with Cd40−/− bone marrow showed lower transaminases (ALT, 175 ± 21 U/L; AST, 827 ± 199 U/L). TNFα is a proinflammatory cytokine produced upon liver damage (34). Consistently, elevated serum TNFα levels were only observed in Cd40−/− tumor-bearing mice after transfer of WT bone marrow cells but not in WT tumor-bearing mice after transfer of bone marrow from Cd40−/− counterparts (Supplementary Fig. S1D).

Agnostic CD40 antibody causes ROS-mediated hepatitis in tumor-bearing mice

ROS produced by mononuclear phagocytes are important mediators of drug-induced hepatotoxicity (35). We studied the role of ROS upon anti-CD40 treatment in tumor-bearing mice using phagocytic NADPH oxidase 2 (Nox2)-deficient mice. Lower transaminases were observed in EL4 tumor-bearing Nox2−/− mice (ALT, 973 ± 474 U/L; AST, 1,700 ± 678 U/L) than WT littermate controls upon agonistic anti-CD40 administration (ALT, 4,452 ± 1,266 U/L; AST, 5,830 ± 1,841 U/L; Fig. 2A). As expected, only moderate ALT elevations were found in tumor-bearing Nox2−/− mice treated with agonistic anti-CD40, possibly due to the direct effect of the antibody on parenchymal cells. In addition, no differences in serum TNFα levels were observed between WT and Nox2−/− tumor-bearing mice upon anti-CD40 agonist treatment (data not shown). Histologic studies revealed milder immune cell infiltration, 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 hepatotoxicity.

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 × 10^6 ± 1.4 × 10^6 vs. tumor-bearing 1.1 × 10^6 ± 1.9 × 10^6, P = 0.052; Fig. 3A).
This increase was significantly higher in the CD11b<sup>+</sup>Gr-1<sup>low</sup> monocytic cell subset (M-MDSC) than in the CD11b<sup>+</sup>Gr-1<sup>high</sup> granulocytic subset (G-MDSC; Supplementary Fig. S3A and S3B).

Apart from CD11b<sup>+</sup>Gr-1<sup>+</sup> cell changes, a moderate decrease of CD3<sup>+</sup>/CD19<sup>+</sup> B cells and an increase of CD11c<sup>+</sup> dendritic cells were observed upon agonistic CD40 antibody injection.

Figure 1. Systemic agonistic anti-CD40 induces immune-mediated liver damage in tumor-bearing (TB) mice. Tumor-free (TF) and tumor-bearing mice received i.p. either 100 µg agonistic CD40 antibody (CD40 Ab) or control IgG (IgG). A, serum ALT and AST levels in C57BL/6 tumor-free and EL4 tumor-bearing mice (n = 6-8 mice/group) were determined 24 hours after antibody injection. Cumulative data expressed as mean ± SEM, representative of three independent experiments. B, serum ALT and AST levels in BALB/c tumor-free mice (n = 4 mice/group) and 4T1 tumor-bearing mice (n = 8 mice/group) were determined 24 hours after antibody injection. Cumulative data from two independent experiments are expressed as mean ± SEM. Representative hematoxylin and eosin staining of liver sections from EL4 tumor-bearing mice 24 hours after IgG (C) or CD40 Ab injection (D). Images show ×20 magnification and yellow bar = 0.2 mm. Tumor-bearing bone marrow chimeric mice (donor → recipient) received i.p. either CD40 Ab or IgG (n = 4 mice/group). Serum ALT and AST (E) levels were measured 24 hours after antibody injection. Cumulative data expressed as mean ± SEM, representative of two independent experiments. n.s., not statistically significant; *, P < 0.05; **, P < 0.01; ***, P < 0.005. Student t test, (E) one-way ANOVA.
Supplementary Fig. S3C–S3G). 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 BALB/c 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 IFN\(\gamma\) release by CD8\(^+\)T cells (Fig. 3H). Finally, significant levels of TNF\(\alpha\) 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
80% were CD11b<sup>+</sup>Gr-1<sup>+</sup>MDSC (Supplementary Fig. S6A and S6B), into CD45.2<sup>+</sup>Cd40<sup>-/</sup>/Cd80<sup>-/</sup>/Cd80<sup>-/</sup>Cd16<sup>-/</sup>ve recipients followed by systemic injection of the agonistic anti-CD40. Although agonistic CD40 antibody did not cause inflammation to regular Cd40<sup>-/</sup>/Cd80<sup>-/</sup>/Cd80<sup>-/</sup>Cd16<sup>-/</sup>ve mice, transfer of tumor-induced hepatic CD45.1<sup>+</sup>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 TNF<sub>a</sub> serum levels was observed (Fig. 4C). Consequently, CD40 ligation on tumor-induced hepatic CD11b<sup>+</sup> cells resulted in higher ALT serum levels compared with Nox2<sup>-/</sup> tumor-induced hepatic CD11b<sup>+</sup> cell transfer (Fig. 4C).

CD40 ligation impairs immunosuppressive function of human CD14<sup>+</sup>HLA-DR<sup>low</sup> MDSC

We isolated human CD14<sup>+</sup>HLA-DR<sup>low</sup> 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<sup>-/</sup> 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<sup>-/</sup> tumor-induced hepatic CD11b<sup>+</sup> cell transfer (Fig. 4C).
human CD14^+ HLA-DR^{low} MDSC in both healthy controls and patients with cancer. This was not observed in CD14^+ HLA-DR^{high} controls (Fig. 5A and B). Similar results were obtained using agonistic anti-human CD40 antibody (Supplementary Fig. S7). Next, we isolated CD14^+ HLA-DR^{low} cells from PBMC and tested their suppressor function after CD40L treatment. CD40 ligation impaired the suppressor function of human CD14^+ HLA-DR^{low} MDSC (Fig. 5C). Interestingly, incubation of PBMC in the presence of CD40L resulted in enhanced ROS production by CD14^+ HLA-DR^{low} cells (Fig. 5D).

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, transaminitis 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 TNF-alpha and IFN-gamma, 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 transaminites 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 hepatic myeloid cells produce ROS upon CD40 ligation, which further biologic evidence by showing that tumor-induced ROS-mediated liver cell damage in our model. We provide AST levels than tumor-bearing littermate controls, suggesting a knockout tumor-bearing mice had lower ALT/AST serum levels.

Our data show that systemic administration of agonistic anti-CD40 increased the accumulation of CD11b+Gr-1low monocytic-like MDSC, CD40 anti-CD40 treatment; (ii) significant accumulation of CD11b+Gr-1low monocytic-like MDSC rather than CD11b+Gr-1high 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. We found that hepatic MDSC accumulate (i) in naive mice after agonistic anti-CD40 treatment; (ii) significant accumulation of CD11b+Gr-1low monocytic-like MDSC rather than CD11b+Gr-1high 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 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), 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.

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+ monocytic-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 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+ monocytic-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 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), 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.

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+ monocytic-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 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+ monocytic-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 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), 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.
Agnostic Anti-CD40 Activates Tumor-Induced CD11b\(^{+}\)Gr-1\(^{+}\) Cells

treated with agnostic anti-CD40, and further studies are clearly needed.

Tumor-induced mouse myeloid cells and human CD14\(^{+}\)HLA-DR\(^{+}\) MDSC both express high arginase levels and can suppress T-cell function through an arginase-dependent mechanism (24, 50). Both our murine and human data clearly suggest that agnostic anti-CD40 treatment may impair the suppressor function of MDSC and would therefore represent a potential novel approach to target MDSC as also recently suggested by others (25, 51).

Immune-related adverse effects in clinical trials have been associated with the systemic use of immune-modulatory drugs (5). Much of anti-CD40 treatment-dependent toxicity can be avoided by local delivery in a slow-release vehicle, maintaining its antitumor effects in animal models (6, 9). However, it is not clear whether such treatment would also induce MDSC maturation. Therefore, further studies are needed to investigate new administration routes and compounds to stabilize and prolong the effect of immune-modulatory compounds with the aim to mitigate immunotherapy-related toxicity and potentially target MDSC.

Overall, our data indicate that liver toxicity caused by systemic CD40 antibody in mice is due to activation of tumor-induced hepatic CD11b\(^{+}\)Gr-1\(^{+}\) cells and provide preliminary evidence suggesting a reprogramming of tumor-induced myeloid cells into proinflammatory myeloid subsets without suppressor function. Finally, our studies not only provide a novel potential explanation for anti-CD40-induced hepatotoxicity observed in early clinical trials, but may also open new opportunities for the targeting of immunosuppressive MDSC in patients with cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: J. Medina-Echeverz, F. Korangy, T.F. Greten

Development of methodology: J. Medina-Echeverz, N. Hawk

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Medina-Echeverz, C. Ma

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Medina-Echeverz, C. Ma, T. Eggert, D.E. Kleiner, T.F. Greten

Writing, review, and/or revision of the manuscript: J. Medina-Echeverz, A. Duffy, T. Eggert, D.E. Kleiner, F. Korangy, T.F. Greten

Study supervision: J. Medina-Echeverz, T.F. Greten

**Grant Support**

This work was supported by the Intramural Research Program of the NCI, NIH.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 29, 2014; revised January 9, 2015; accepted January 22, 2015; published OnlineFirst January 30, 2015.

**References**


