Activation of 4-1BB on Liver Myeloid Cells Triggers Hepatitis via an Interleukin-27–Dependent Pathway

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Abstract

Purpose: Agonist antibodies targeting the T-cell costimulatory receptor 4-1BB (CD137) are among the most effective immunotherapeutic agents across preclinical cancer models. In the clinic, however, development of these agents has been hampered by dose-limiting liver toxicity. Lack of knowledge of the mechanisms underlying this toxicity has limited the potential to separate 4-1BB agonist–driven tumor immunity from hepatotoxicity.

Experimental Design: The capacity of 4-1BB agonist antibodies to induce liver toxicity was investigated in immunocompetent mice, with or without coadministration of checkpoint blockade, via (i) measurement of serum transaminase levels, (ii) imaging of liver immune infiltrates, and (iii) qualitative and quantitative assessment of liver myeloid and T cells via flow cytometry. Knockout mice were used to clarify the contribution of specific cell subsets, cytokines, and chemokines.

Results: We find that activation of 4-1BB on liver myeloid cells is essential to initiate hepatitis. Once activated, these cells produce interleukin-27 that is required for liver toxicity. CD8 T cells infiltrate the liver in response to this myeloid activation and mediate tissue damage, triggering transaminase elevation. FoxP3+ regulatory T cells limit liver damage, and their removal dramatically exacerbates 4-1BB agonist–induced hepatitis. Coadministration of CTLA-4 blockage ameliorates transaminase elevation, whereas PD-1 blockage exacerbates it. Loss of the chemokine receptor CCR2 blocks 4-1BB agonist hepatitis without diminishing tumor-specific immunity against B16 melanoma.

Conclusions: 4-1BB agonist antibodies trigger hepatitis via activation and expansion of interleukin-27–producing liver Kupffer cells and monocytes. Coadministration of CTLA-4 and/or CCR2 blockade may minimize hepatitis, but yield equal or greater antitumor immunity. Clin Cancer Res; 24(5); 1–14. ©2018 AACR.

Introduction

The transformative efficacy of checkpoint blockade immunotherapy for the treatment of melanoma has revolutionized the field of oncology and initiated a new era of immune-targeted therapeutics (1, 2). Beyond blockade of T-cell coinhibitory receptors, agonist antibodies that activate tumor necrosis factor superfamily receptors have demonstrated significant therapeutic potential in both preclinical models and clinical trials (3). Among these agonists, activators of the costimulatory receptor 4-1BB (CD137) have demonstrated exceptional potency across multiple preclinical tumor models, as well as the capacity to elicit objective clinical responses in patients with diverse cancers (4, 5).

In addition to mediating tumor regressions, releasing the “brakes” on T-cell responses with checkpoint blockade can also trigger T-cell responses targeting normal self-tissues known as immune-related adverse events (IRAE). These IRAE can be severe and even life-threatening, but are readily managed with timely steroid intervention (6). 4-1BB agonist antibodies, by contrast, can effectively treat autoimmunity in a variety of murine models and may even ameliorate CTLA-4 antagonist antibody-induced IRAE (7, 8). Despite this, these agents induce a unique spectrum of on-target adverse events ranging from mild to moderate hematologic perturbations, up to high-grade transaminitis and potentially fatal hepatotoxicity (9, 10).

We sought to elucidate the underlying mechanisms by which 4-1BB antibody therapy promotes liver damage, and to explore potential avenues to uncouple augmentation of antitumor immunity from hepatitis. Results presented here demonstrate that 4-1BB agonist–induced hepatotoxicity initiates at the myeloid level through activation of liver-resident Kupffer cells. Moreover, we find that the inflammatory cytokine interleukin 27 (IL27), released from these cells in response to activation, is critically required for hepatic damage. We further show that, in contrast to CD40 agonist–induced acute hepatotoxicity, 4-1BB agonist...
Translational Relevance

4-1BB (CD137) agonist antibodies are among the most potent immunotherapeutic agents across a broad spectrum of preclinical cancer models. Despite entering clinical trials before any PD-1–blocking agents, no 4-1BB antibody has advanced beyond early phase II studies. Although 4-1BB agonist antibodies have elicited objective clinical responses in patients with advanced solid tumors, their development has been hampered by dose-limiting, high-grade hepatitis. Lack of understanding of the mechanisms underlying this liver toxicity has limited efforts to neutralize or manage it, while preserving the capacity of 4-1BB agonists to activate tumor immunity. We describe a pathway by which 4-1BB activation on liver myeloid cells triggers inflammatory cytokine production, particularly interleukin-27. This then facilitates activation and costimulation of cytotoxic CD8 T cells, which mediate liver tissue injury and transaminase elevation. We show that coblockade of CTLA-4 and/or CCR2 can ameliorate hepatitis without impairing tumor immunity, providing a path to clinical translation.

Therapeutic antibodies

T-cell costimulatory modulating antibodies were purchased from BioXcell: 4-1BB [3H3 (Rat IgG2a), 250 μg/dose], CTLA-4 [9D9 (mouse IgG2b) or 9H10 (Syrian Hamster Ig), 100 μg/dose], PD-1 [RMP1-14 (Rat IgG2a), 250 μg/dose], and CD40 [FGK4.5 (Rat IgG2a), 100 μg/dose]. All doses indicate quantity administered per injection. The mouse CTLA-4 antibody 9D9 engages the mouse IgG2b receptor, which gives it a low-to-moderate ADCC capacity similar to the human CTLA-4 antibody ipilimumab (human IgG1). The mouse 4-1BB antibody 3H3 is more similar to the human antibody uralumab as it exhibits strong agonist activity, while utomilumab is a weaker agonist. RMP-14 is a purely blocking antibody for PD-1 with weak Fc receptor binding similar to the human PD-1 antibodies pembrolizumab and nivolumab which are human IgG4.

Antibody treatment and liver enzyme analysis

Antibodies were given i.p. for 3 doses every 3 days. On day 16 after initiation of therapy, mice were bled and serum levels of aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (AP) were measured by the MDACC Veterinary Diagnostic Laboratory. Mice were sacrificed; livers were perfused with PBS and harvested for immune infiltrates.

Tumor therapy

Wild-type (WT), CCR2−/−, CXCR3−/−, or CCR5−/− mice were implanted s.c. with 3 × 105 B16-Ova cells on the flank as described (11, 12). On days 3, 6, and 9 mice received α4-1BB i.p., and a mixture of irradiated FVAX and B16-Ova s.c. on the opposite flank as described (12). On day 19, mice were sacrificed and tumors and perfused livers were harvested for analysis of immune infiltrates.

Treg depletion and adoptive transfer

Mice bearing the diphtheria toxin (DT) receptor driven by the Foxp3 promoter (Foxp3-DTR) were administered DT at 10 μg/kg 1 day prior to α4-1BB and every 3 days thereafter until sacrifice. Alternately, CD4+CD25+CD3+ cells were FACs sorted from naive spleens and 5 × 105 cells were injected into host mice 1 day prior to immunotherapy.

Myeloid cells were adoptively transferred by magnetically sorting bone marrow–derived monocyes using a monooyte isolation kit (Miltenyi Biotec). Sorted cells (CD45.2) were adoptively transferred at 2 × 106 cells/mouse into congenically marked (CD45.1) mice before initiation of therapy.

Cell isolation

Livers were perfused with PBS, and tumors were harvested for analysis of immune infiltrate as described (13, 14).

Flow cytometry analysis

Samples were fixed using the Foxp3/Transcription Factor Staining Buffer Set (Thermo) and then stained with up to 16

Antibody therapy induces a chronic hepatotoxicity characterized by dense and persistent T-cell infiltration in the hepatic portal zones. This infiltrate is dominated by CD8+ T cells, which are the primary effectors of liver tissue injury. CD4+Foxp3+ regulatory T cells (Treg), on the other hand, act to maintain tissue tolerance and limit α4-1BB–induced hepatic damage. Treg ablation severely exacerbates 4-1BB agonist liver inflammation and abrogates the capacity of CTLA-4 blockade to ameliorate transaminitis. Finally, we show that chemotaxis of immune cells into the liver is a critical step in the progression of liver injury. While hepatic immune responses following 4-1BB agonist therapy rely heavily on the chemokine receptors CCR2 and, less so, on CXCR3, these receptors appear to be largely dispensable for antimelanoma immunity in the same animals. These data suggest that differential trafficking requirements for the liver and tumor microenvironments may be exploited to increase the tumor selectivity of 4-1BB agonist antibody immunotherapy.

Materials and Methods

Animals

Male (6-week) C57BL/6 mice were purchased from Taconic Biosciences. 4-1BB−/−, EBl3−/−, IL27 receptor alpha−/−, β2M−/−, MHCII−/−, Foxp3-DTR, CXCR3−/−, CCR2−/−, and CCR5−/− mice were purchased from The Jackson Laboratory. All procedures were conducted in accordance with the guidelines established by the UT MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Cell lines and reagents

B16 melanoma, B16-Fli3-ligand (FVAX), and B16-Ova were obtained/created and cultured as described previously (11, 12). The BV421-labeled H2-Kb epitope OVA257-264 (SIINFEKL)-containing tetramer was acquired from the Tetramer Core Facility at the National Institute of Health (Emory University, Atlanta GA).

immune ablation and reconstitution

C57BL/6 mice or 4-1BB−/− mice were sublethally irradiated (500 rad) using a Cesium-137 irradiator. One day later, splenic lymphocytes were isolated using CD90.2 magnetic beads (Miltenyi Biotec) and injected i.v. at 2 × 106 cells/mouse into irradiated hosts.

Antigenic analysis of immune infiltrates

Antibodies were given i.p. 3 times every 3 days. On day 16 after initiation of therapy, mice were bled and serum levels of aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (AP) were measured by the MDACC Veterinary Diagnostic Laboratory. Mice were sacrificed; livers were perfused with PBS and harvested for immune infiltrates.

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antibodies at a time from Biolegend, BD Biosciences, and Thermo. Flow cytometry data were collected on an 18-color BD LSR II cytometer and analyzed in FlowJo (TreeStar).

Immunohistochemistry
Each liver lobe was collected and formalin fixed separately for ≥24 hours. Tissues were then paraffin embedded (FFPE), sectioned and stained for hematoxylin and eosin (H&E) and IHC for CD8 and F4/80, at the MDACC Research Histology, Pathology, and Imaging Core at Science Park. Two sections were generated from the left lateral lobe at the widest dimension, and stained by H&E. H&E sections were evaluated by semiquantitative scoring based on the number of inflammatory and necrotic cells in the portal triad, central vein, or parenchyma. A score of 0 or nil indicates no inflammation; score 1, minimal inflammation, <15 inflammatory cells around portal triad, central vein, or in parenchyma; score 2, mild inflammation, >15 inflammatory cells around portal triad, central vein, or in parenchyma; score 3, moderate inflammation, >30 inflammatory cells around portal triad, central vein, or in parenchyma, and score 4: severe inflammation, approximately >50 cells around portal triad, central vein, or in parenchyma.

Two sections per animal per group were stained with the following immunohistochemical stains: CD8 and F4/80. The number of CD8+ and F4/80+ cells in the liver, both at the perivascular zones (central vein or portal area) and in the parenchyma, was counted separately in a microscopic field at 20× magnification. Four areas with the most abundant infiltration were selected for both areas, and the average number per animal was calculated as described in Peng and colleagues (15).

Immunofluorescence staining and imaging
Tissues were collected and flash frozen in liquid nitrogen. The frozen tissues were embedded in Tissue-Tek OCT Compound (Sakura) and sectioned at the MD Anderson Histology Core. The sectioned tissues were fixed with acetone for 10 minutes, then stained with various antibodies and mounted in Prolong Gold (Invitrogen). Confocal imaging was performed using a TCS SP8 laser-scanning confocal microscope equipped a 20× objective (HCPl, APO 20×/0.70 NA), Leica Microsystems) with lasers for excitation at 405, 458, 488, 514, 543, and 633 nm wavelengths (Leica Microsystems, Inc.).

Real-time PCR
Liver myeloid subpopulations were sorted as shown (Supplementary Fig. S1) at the MD Anderson Flow Cytometry and Cellular Imaging Core Facility (FCCIF). Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the SuperScript IV Reverse Transcriptase kit (Thermo). Taqman real-time PCR was performed on a ViiA 7 Real Time PCR System (Applied Biosystem) as previously described (13, 14). Levels of il27-p28, ifng, and tnfa were expressed as the fold change using the ΔΔCt method.

Cytometric bead array
Bone marrow–derived monocytes were isolated from WT mice using a Monocyte Isolation Kit (Miltenyi Biotech) and were stimulated in vitro with α4-1BB (3H1) antibody for 48 hours. Cytokine release was quantified using a Th1/Th2/Th17 Cytometric Bead Array kit (BD) as per the manufacturer’s instructions.

Statistical analysis
All statistics were calculated using Graphpad Prism Version 6 for Windows. Statistical significance was determined using a two-sided Student t test applying Welch correction for unequal variance. Graphs show mean ± standard deviation unless otherwise indicated. P values less than 0.05 were considered statistically significant.

Results
Disparate effects of CTLA-4 and PD-1 checkpoint blockade on α4-1BB–mediated hepatotoxicity
To determine the potential for currently approved checkpoint blockade antibodies (αCTLA-4, αPD-1) to ameliorate 4-1BB agonist antibody-induced liver pathology, mice were treated with three administrations of checkpoint antibody, α4-1BB alone, α4-1BB in combination with αCTLA-4 or αPD-1, or triple combination therapy. At the peak of hepatic injury, 16 days after the initiation of treatment (Supplementary Fig. S1A), mice were bled and serum was analyzed for liver transaminases including alanine aminotransferase (ALT; reference mean 26.5 ± 5) and aspartate aminotransferase (AST; reference mean 43.2 ± 9.5; ref. 16). As noted previously, coadministration of αCTLA-4 significantly decreased serum transaminase levels compared with α4-1BB monotherapy (8), whereas dual therapy with α4-1BB and αPD-1 significantly increased transaminase levels (Fig. S1A; ref. 17). The protective effect of αCTLA-4 therapy was lost when given in combination with both α4-1BB and αPD-1, suggesting that exacerbation of hepatitis by αPD-1 dominates over the capacity of αCTLA-4 to limit it. As triple combination therapy failed to alleviate hepatic damage, we sought to define the cellular mechanisms by which CTLA-4 blockade acted to limit α4-1BB hepatotoxicity.

Figure 1.
Combination immunotherapy augments α4-1BB–mediated hepatotoxicity. Mice were administered α4-1BB, αCTLA-4, or αPD-1 antibodies alone or in combination within 3-day intervals (days 0, 3, and 6). Mice were bled 16 days after initiation of therapy and sacrificed to measure liver immune infiltrates by flow cytometry. A, Serum levels of ALT and AST were measured upon sacrifice as units of enzyme/liter of blood. B, Immune infiltrates within perfused livers of treated mice were measured by flow cytometry. Percentage of CD3+ T cells was calculated as a percentage of CD3+ cells. Frequency of CD8+ T cells was calculated as a percentage of CD3+ cells. C, H&E staining or IHC targeting CD8 (D) was performed on sectioned liver tissues from treated mice 16 days after initiation of therapy. E, Sections were assigned a clinical score by a pathologist based on the number of inflammatory cells in the portal triad, central vein, or parenchyma, and F, CD8+ infiltration was enumerated per section. G, Mice administered either α4-1BB or αCD40 agonist antibodies were bled 8 or 16 days after initiation of therapy, and serum levels of ALT and AST were analyzed. H, Mice were administered either αCD40 agonist antibodies alone or in combination with αCTLA-4 blockade. Mice were then bled at the peak of αCD40-mediated liver damage (DB) in order to assess serum transaminase levels. Each point in A and B represents an individual mouse. Micrographs in C and D were imaged at 20× magnification. Data were pooled from ≥3 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student t test applying Welch correction for unequal variance. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
4-1BB agonist administration drove robust CD3+ T-cell infiltration of the liver including 2-fold increases in cytotoxic CD8 T cells relative to untreated animals or those receiving CTLA-4 blockade (Fig. 1B; Supplementary Fig. S1B), but did not significantly impact infiltration of bulk CD4+ T cells (CD4+ CD3+) or CD4+ effector T cells (CD4+ CD3+ FoxP3+) (Supplementary Fig. S2A and S2B). Functionally, the majority of these infiltrating T cells bore the recently defined Eomesodermin "KLRG1" signature of the cytotoxic ThEO (CD4) and TcEO (CD8) phenotype that are critical for antitumor immunity by exhibiting elevated cytotoxicity compared with their Th1/Tc1 counterparts and likely play a significant role in mediating liver damage (Supplementary Fig. S2C–S2E; refs. 14, 18–20). Further, the addition of CTLA-4 blockade to 4-1BB treatment reduced the frequency of T-cell infiltration into the liver versus 4-1BB alone (Fig. 1B). Whereas the overall CD3 density was reduced in 4-1BB/αCTLA-4 combination–treated animals, no changes in the CD4 and CD8 frequencies within the infiltrating T-cell pool, nor in the percentage of cells adopting the ThEO/TcEO phenotype were observed (Fig. 1B; Supplementary Fig. S2D and S2E). Consistent with the overall decrease in T-cell infiltration, inflammatory foci (Fig. 1C) and clusters of CD8 T cells in the liver parenchyma also decreased when αCTLA-4 was coadministered with 4-1BB, but were exacerbated by triple combination therapy (Fig. 1D and E). Overall, αCTLA-4 coadministration with 4-1BB significantly decreased the severity of inflammation, necrotic regions, and CD8 T-cell infiltration in liver parenchyma as indicated by a reduced pathology score (Fig. 1E and F).

To test whether the ability of CTLA-4 blockade to reduce liver pathology was specific for 4-1BB agonist therapy, we also tested αCTLA-4 in combination with antibodies targeting the TNF receptor CD40. Costimulation through CD40 induces an acute and transient hepatic injury that peaks within a week of antibody administration and declines thereafter, whereas 4-1BB agonists induce a chronic and persistent hepatic pathology as measured by maintained elevation of serum transaminases over the 16-day study (Fig. 1G). Further, in contrast to 4-1BB, αCD40-induced liver damage was not ameliorated by coadministration with αCTLA-4 (Fig. 1H).

These data suggest that 4-1BB agonist antibodies mediate chronic liver pathology through a mechanism distinct from CD40 activation. Although CTLA-4 blockade can ameliorate 4-1BB agonist–induced hepatitis through reduction of T-cell infiltration; this mechanism fails to impact liver injury resulting from αCD40 or α-1BB/αPD-1 combination therapy.

4-1BB agonists initiate liver pathology through activation of liver-resident myeloid cells

Given the differential liver toxicities associated with 4-1BB agonists and CD40 agonists, we sought to uncover the relative contribution of the myeloid and T-cell pools to 4-1BB agonist–induced liver damage. Whereas CD40 is exclusively expressed by myeloid cells (21), 4-1BB can be expressed on both T-cell, NK-cell and myeloid populations (4, 14, 22, 23), and the relative contribution of each of these to liver pathology remains undefined.

To reveal the relative contribution of the myeloid versus lymphocyte compartments with 4-1BB–induced hepatotoxicity, WT or 4-1BB−/− mice were administered a sublethal dose of radiation sufficient to eliminate their endogenous lymphocytes. Twenty-four hours after irradiation, splenic lymphocytes from WT or 4-1BB−/− mice were transferred into irradiated WT or 4-1BB−/− hosts. In this way, ablation of the lymphoid pool, but not the radioresistant myeloid pool, allowed us to specifically target 4-1BB on either T cells or myeloid cells. Mice then received 4-1BB agonist therapy as previously described. Mice receiving WT to WT splenocyte transfers (myeloid 4-1BB+, lymphocyte 4-1BB−) clearly manifested ALT elevation in response to 4-1BB agonist antibody treatment compared with WT to WT transfers administered isotype control antibodies or 4-1BB−/− mice receiving 4-1BB−/− cells in conjunction with 4-1BB (Fig. 2A), while AST elevation, which is always less affected by 4-1BB, showed modest elevation as well (Supplementary Fig. S3A). WT mice that received splenocytes from 4-1BB−/− mice (myeloid 4-1BB+, lymphocyte 4-1BB−) were not significantly protected against ALT elevation, but did show reduced elevation of AST. On the other hand, 4-1BB−/− mice receiving splenocytes from WT mice (myeloid 4-1BB+, lymphocyte 4-1BB−) were fully protected from ALT elevation and showed no significant elevation of AST relative to mice lacking 4-1BB only on T cells. Thus, when 4-1BB was absent from the myeloid compartment, 4-1BB could no longer trigger hepatotoxicity, suggesting a requirement for myeloid 4-1BB activation to initiate a liver injury.

Figure 2.
Administration of 4-1BB agonist antibodies initiates liver pathology through activation of liver-resident myeloid cells. A, Mice were sublethally irradiated (500 rad) before administration of 2 × 10^6 CD90− splenocytes. WT mice received splenocytes from either WT mice (WT→WT) or 4-1BB−/− mice (4-1BB−/−→WT), and 4-1BB−/− mice received splenocytes from WT mice (WT→4-1BB−/−) or from 4-1BB−/− mice (4-1BB−/−→4-1BB−/−). Mice were subsequently treated with 3 rounds of isotype control or 4-1BB immunotherapy. Treated mice were then bled 16 days after the first administration of therapy, and serum ALT was measured. B, Frequency of F4/80− myeloid infiltration into perfused livers based on flow cytometry of lymphoid-replete WT mice administered α4-1BB therapy either alone or in combination with αCTLA-4 checkpoint blockade. Myeloid infiltration shown as the percentage of F4/80+ cells as a fraction of total CD45+ cells. C, Immunohistochemistry staining for F4/80 was performed on seconed liver tissues from treated mice 16 days after initiation of therapy. D, Quantification F4/80+ cellular infiltrates based on IHC staining of liver sections. Individual F4/80+ cells were enumerated within the liver parenchyma or perivascular space. E, Confocal imaging of myeloid immune infiltrates in naive or α4-1BB–treated livers 16 days after initiation of treatment. F, Phenotypic characterization of congenitally marked, adoptively transferred bone marrow–derived myeloid cells into perfused livers and blood based on flow cytometry of mice administered α4-1BB therapy. G, Frequency of inflammatory/activation markers based on flow cytometry of perfused livers from treated mice based on 3 subsets of liver-resident macrophages: CD11b−CD68− cytokine-producing/phagocytic Kuffer cells, CD11b+CD68+ cytokine-producing/phagocytic Kuffer cells, and CD11b−CD68+ phagocytic Kuffer cells. H, Gene expression from individual myeloid populations was calculated at day 7 after treatment initiation using real-time PCR analysis with gapdh as the endogenous control. Each point in A and B represents an individual mouse. Micrographs in C were imaged at 20× magnification. Micrographs in E were imaged using a 20× air objective. Insets magnified using 2× magnification. Gene expression was calculated using Taqman primers via the ΔΔCt method. Data were pooled from ≥2 experiments with 5 mice per group. Bars represent mean ± SEM. Statistical significance was calculated using a two-sided Student t test applying Welch correction for unequal variance. ns, not significant; *, P < 0.05; **, P < 0.01; ††, P < 0.001; †††, P < 0.0001.
inflammatory cascade. The absence of 4-1BB on T cells did not appear deterministic for liver inflammation, but the modest reductions in transaminases relative to WT mice suggested a contributory role for 4-1BB on T cells as well.

Given our prior data, we investigated the role of myeloid cells in initiating α4-1BB–induced liver pathology. We found that, in comparison with untreated livers, 4-1BB therapy increased the frequency of F4/80+ macrophages within the liver parenchyma, which was significantly reduced by combining αCTLA-4 with α4-1BB (Fig. 2B–D). Interestingly, combination therapy favored accumulation of F4/80+ cells within the perivascular space compared with infiltration into the tissue parenchyma (Fig. 2D). The expanded liver macrophages consist of tissue-resident Kupffer cells, defined by expression of the adhesion receptor F4/80, that remain relatively quiescent within healthy liver, are replenished by bone marrow-derived myeloid precursors or via low-level homeostatic proliferation, and are functionally and phenotypically distinct from circulating CD11b+ F4/80+ monocytes (24). Further, Kupffer cells can be subclassified into populations of CD11b+ CD68+ myeloid cells specialized for cytokine production, CD11b+CD68+ phagocytic macrophages, and CD11b+CD68− cells with intermediate phagocytic activity and cytokine expression (25). In naïve mice, we were only able to detect clear 4-1BB expression on monocytes by flow cytometry (Supplementary Fig. S3B); however, 4-1BB expression was detected on both F4/80+ monocytes and on a small percentage of F4/80+ Kupffer cells in situ by immunofluorescence (Fig. 2E). The Kupffer cell phenotype is sensitive to disruptive procedures used to prepare livers for flow cytometry, likely explaining the lower resolution of flow cytometry. Both methods, however, showed that 4-1BB was readily induced on Kupffer cells by inflammatory cytokines such as TNFα, which are plentiful during α4-1BB–induced liver injury, with flow cytometry confirming the CD11b+CD68+ and CD11b+CD68− subpopulations as the primary targets (Fig. 2E; Supplementary Fig. S3C). To assess the origin of these Kupffer cell populations, as well as the plasticity of infiltrating bone marrow–derived monocytes, we adoptively transferred congenically labeled bone marrow myeloid progenitor cells and administered α4-1BB to the recipient mice. In response to 4-1BB activation, these monocytes expanded in the blood and infiltrated the liver (Supplementary Fig. S3D). A majority of these liver-infiltrating cells remained phenotypically monocytes (CD11b+ F4/80−); however, some capacity to differentiate into CD11b+CD68+ and CD11b+CD68− subpopulations of Kupffer cells was observed (Fig. 2F). This is consistent with the recent literature showing that while most Kupffer cells originate from embryonically derived erythro-myeloid progenitor (EMP) cells, some capacity of bone marrow–derived monocytes to replenish these populations does exist (26, 27).

Based on these findings, we hypothesize that bone marrow–derived monocytes infiltrate the liver and, in response to 4-1BB activation, initiate a cascade of inflammatory cytokine production (Supplementary Fig. S3E), which triggers 4-1BB upregulation by resident Kupffer cells allowing them to respond in turn to the agonist antibody (Supplementary Fig. S3C). Our data, however, do not rule out a minor contribution of 4-1BB+ monocytes differentiating into resident cells with a Kupffer phenotype themselves and contributing to the response directly.

Further, all three Kupffer cell subsets showed signs of activation in response to 4-1BB agonist antibody (Fig. 2G). Increases in the CCR5+ fraction of the CD11b+CD68+ and CD11b+CD68− subpopulations by approximately 2-fold suggest that these cells are either new emigrants or derived from them, or, alternatively that they are redistributing within subcompartments of the liver. Both possibilities are consistent with increased infiltration into the perivascular space that we observed (28, 29). CCR5 expression decreased, however, on the CD11b+CD68+ subset, which may be a result of receptor downregulation by recent emigrants from the bone marrow as we observed no evidence of elevated in situ proliferative expansion by Ki67. Moreover, all three subsets of F4/80+ cells increased MHC-II expression, further suggesting that these populations are activated by 4-1BB antibody consistent with the published literature demonstrating that this activation promotes enhanced costimulatory capacity (14, 22).

We next sought to confirm the ability of the cytokine-producing myeloid populations to mediate liver damage during the course of α4-1BB therapy, as well as to determine what effector molecules these populations produce to mobilize immune responses leading to hepatic damage. Within the F4/80-positive population, CD68+ (F4/80+CD11b+CD68+), CD11b+ (F4/80+CD11b+CD68+), and CD11b−CD68− (F4/80+CD11b+CD68−) cells as well as CD11b+ F4/80− monocytes were FACS sorted on day 7 from the livers of treated mice (Supplementary Fig. S1), and RNA was isolated from each population for quantitative real-time PCR. We found that, compared with αCD40 treatment which induced significant activation and IFNγ production in CD11b+CD68+ Kupffer cells, the F4/80+CD11b+CD68− and F4/80+CD11b−CD68+ myeloid cells were the predominant cytokine producers with little or no contribution from the CD11b− subset within the livers of α4-1BB–treated mice. Within the two CD11b−CD68+ subsets, we observed approximately 20-fold increased expression of IL27-p28 following 4-1BB agonist therapy compared with treatment-naïve mice. In contrast, the CD11b+CD68− subset was the primary source of interferon-γ (Fig. 2H). Moreover, both CD11b+ subsets of Kupffer cells produced the majority of TNFα. Notably, the cytokine producing subsets of myeloid cells produced less IL27 and TNFα in mice receiving the α4-1BB/αCTLA-4 combination therapy compared with mice receiving α4-1BB monotherapy. While the CD11b+CD68− subset demonstrated roughly 50-fold increases in IL27-p28 expression relative to its baseline level during α4-1BB/αCTLA-4 combination therapy, the delayed cycle within in which transcripts were detected (cycle 37 vs. cycle 26 for the cytokine-producing subsets) suggests that the actual quantity of transcript present in these cells was extraordinarily small.

Together, these data suggest α4-1BB–mediated inflammatory hepatotoxicity initiates at the myeloid level via activation of tissue-resident Kupffer cells and, potentially, infiltrating monocytes. All three subsets of Kupffer cells, and to a lesser extent monocytes, showed signs of activated antigen presentation, and both CD11b+ cytokine-producing subsets increased production of IL27. Coadministration of CTLA-4 blockade reduced inflammatory cytokine production in these subsets, consistent with the reduced transaminase elevation observed in those mice.

IL27 is a critical regulator of liver inflammation
In addition to the above, we previously reported that IL27 acts to polarize T cells to the cytotoxic ThE0/ThE0 phenotype (14), and therefore hypothesized that it may play a role in triggering α4-1BB–induced hepatic damage. To evaluate the contribution
of IL27 to immune-mediated hepatotoxicity, mice lacking the Eb3 subunit of IL27 (EBI3−/−) or mice lacking the IL27 receptor alpha subunit (IL27Ra−/−) were treated with α4-1BB therapy followed by analysis of transaminase levels. Compared with WT mice, EBI3−/− and IL27Ra−/− mice treated with 4-1BB agonists failed to develop liver damage as measured by serum ALT and AST (Fig. 3A). Remarkably, the high-grade elevation of liver transaminases resulting from triple combination α4-1BB/αCTLA-4/αPD-1 therapy was also nearly completely abrogated in EBI3−/− mice. Moreover, abrogation of the IL27 pathway did not significantly affect basal 4-1BB expression nor TNFα-induced expression on liver-resident myeloid populations (Supplementary

Figure 3. IL27 is a critical regulator of 4-1BB agonist-induced liver inflammation. WT mice or mice lacking the Ebi3 subunit of the IL27 cytokine complex (EBI3−/−) or the IL27 receptor alpha subunit (IL27Ra−/−) were treated for three rounds of α4-1BB agonist immunotherapy before analysis of serum transaminase levels and hepatic immune infiltrates 16 days after initiation of treatment. A, Serum levels of ALT and AST were measured upon sacrifice as units of enzyme/liter of blood volume. B, Immune infiltrates within perfused livers of treated mice were quantified by flow cytometry. Frequency of CD3+ cells was calculated as a percentage of total CD45+ cells in the liver. C, Frequency of CD8+ T cells was calculated as a percentage of CD3+ cells. Total numbers of cells were taken as number of CD3+ or CD3+CD8+ cells within perfused livers. D, Quantification of percentage and total numbers of TcEO T cell infiltration within the livers of treated mice. Frequency of TcEO was calculated based on the percentage of CD3+CD8+ T cells expressing Eomesodermin (Eomes) and KLRG1. Each point within each graph represents an individual mouse. Data were pooled from ≥2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student's t test applying Welch correction for unequal variance. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Fig. S4A and S4B), suggesting that EBI3−/− mice were equally capable of receiving 4-1BB signal.

In mice lacking the IL27/IL27R pathway, CD3+ T-cell infiltration of the liver was reduced (Fig. 3B) as were both the frequency and density of cytotoxic CD8+ cells (Fig. 3C). Further, the frequency of CD4 effector T cells appeared minimally affected by knockout of the IL27 pathway (Supplementary Fig. S4C). While the percentage of CD4+Eomes+KLRG1+ ThEO phenotype cells (Supplementary Fig. S4D) and CD8+ TeEO phenotype T cells was minimally affected by loss of IL27, the total numbers of the highly inflammatory TeEO population within liver infiltrates were significantly diminished absent functional IL27 signaling (Fig. 3D).

Taken together, these data demonstrate a critical requirement for the inflammatory cytokine IL27 in mediating 4-1BB agonist antibody-induced hepatotoxicity as well as for recruitment and/or expansion of hepatic T cells into the liver.

Tregs restrict 4-1BB agonist antibody–induced liver pathology

Given the ability of myeloid cells to activate T-cell responses, coupled with the capacity of IL27 to act as an inflammatory mediator of hepatic damage with pleiotropic effects on helper T-cell polarization, Treg suppression, and T-cell trafficking (30–32), and the prolonged inflammatory response induced by 4-1BB (Fig. 1C), we investigated the role of T cells in propagating 4-1BB–mediated liver damage. To assess the relative contribution of the T-cell pool in mediating hepatotoxicity, we administered 4-1BB to mice lacking the β2 microglobulin subunit of the major histocompatibility (MHC) I complex (β2M−/−) or mice lacking all H2-A/E MHC genes (MHCII−/−). These mice are deficient in antigen presentation to CD8 and CD4 T cells respectively, leading to a failure of these cells to complete thymic positive selection and enter the periphery. Even though these mice exhibited similar patterns of 4-1BB expression compared with WT mice (Supplementary Fig. S5A and S5B), elevation of liver ALT and AST levels was completely abrogated in 4-1BB–treated β2M−/− mice, confirming the role of CD8+ T cells in mediating the bulk of the liver damage (Fig. 4A; ref. 9). To separate the possibilities that this effect may be due to absent CD8 T-cell responses and/or to defective antigen presentation, mice were sublethally irradiated and CD8+ splenocytes from WT mice were transferred into β2M−/− mice. We hypothesized that if the lack of CD8 T cells in these mice was the sole cause of the abrogated hepatotoxicity, then supplying WT CD8+ T cells would reinstitute toxicity. Interestingly, supplementation of WT CD8+ T cells into β2M−/− mice did not abrogate the resistance of these animals to liver damage when challenged with 4-1BB antibody (Fig. 4B). This suggests that not only are CD8 T cells required to effect 4-1BB agonist–induced liver injury, but that antigen presentation on MHC Class I is also necessary. This further indicates that hepatitis-inducing CD8 T cells are being activated by 4-1BB–activated myeloid cells in an antigen-specific manner. Intriguingly, impairing the CD4 response in MHCII−/− mice significantly escalated liver damage, denoted by approximately 1.5–2-fold increases in serum AST (176 vs. 87; P = 0.0008) and ALT (108 vs. 84; P = 0.0244) levels in MHCII−/− mice compared with 4-1BB–treated WT mice (Fig. 4A).

We next hypothesized that exacerbation of hepatotoxicity in MHCII−/− mice stemmed, not from dysregulation of effector T-cell responses, but from elimination of Tregs leading to loss of immune homeostasis in the liver. We made the related observation that there was a 2-fold increase in the fraction of Foxp3+ Tregs in the livers of 4-1BB compared with untreated mice (Fig. 4C), suggesting that Treg expansion might be acting to limit hepatitis. To validate a role for Tregs in limiting 4-1BB–induced liver toxicity, we treated mice expressing the DT receptor (DTR) under control of the Foxp3 promoter (Foxp3-DTR) in which Foxp3+ Tregs can be depleted upon administration of DT. Briefly, DT was administered 2 days before 4-1BB therapy and continued until the end of treatment for complete and sustained Treg depletion. Treg depletion was successful based on analysis of blood 3 days before serum analysis (Supplementary Fig. S5C). Consistent with our hypothesis, depletion of Tregs significantly aggravated 4-1BB induced liver damage, increasing AST and ALT levels 5–6-fold, and eliminating the ability of αCTLA-4 to dampen liver damage (Fig. 4D). This effect was not due to administration of DT, as DT alone did not significantly impact transaminase levels. Moreover, Treg adoptive transfer prior to therapy limited transaminase elevation, suggesting that Tregs are critical suppressors of inflammation during 4-1BB treatment. Of note, while the CTLA-4 antibodies used here are capable of depleting Tregs in the context of tumor microenvironments, they do not deplete peripheral Tregs, and may sometimes expand them, due to the low densities of the FcyRIV receptor in these tissues (33).

Taken together, these data suggest a critical role of CD8 T-cell activation in mediating 4-1BB liver damage. Antigen presentation was also required, suggesting hepatogenic CD8 T cells are liver tissue-antigen specific. Further, Treg cells play a critical role in protecting the liver from CD8-mediated injury downstream of 4-1BB.

CCR2 and CXCR3 are differentially required for liver and tumor T-cell trafficking

Given the ability of IL27 to induce chemokine receptor expression (34, 35), the reduced immune infiltrate in the liver in the absence of IL27, and the reduced myeloid presence in mice treated with 4-1BB/αCTLA-4 cotherapy, we hypothesized that 4-1BB agonist therapy might alter T-cell trafficking patterns into the tissue via chemokine modulation. Given the differential expression patterns of chemokine receptors on T cells capable of homing into tumor tissue versus liver (29, 36), we sought to determine whether antitumor immunity could be separated from hepatitis based on differential homing. We challenged either WT, CCR2−/−, CXCR3−/−, or CCR5−/− mice subcutaneously with 3 × 10⁶ murine B16 melanoma cells expressing the ovalbumin antigen (B16-Ova). Mice were then treated with 4-1BB agonist and assessed for serum transaminase elevation and infiltration. CXCR3 is critical for driving IFNγ-dependent T-cell trafficking into tumors, while CCR5 remains the predominant trafficking mechanism into the liver; however, CXCR3 can regulate liver chemotaxis in response to injury (37). CCR2, in contrast, minimally impacts T-cell trafficking to liver even in the context of viral infection. Intriguingly, following 4-1BB agonist antibody therapy, CCR2−/− mice exhibited significantly reduced AST and ALT serum levels, while CXCR3−/− mice showed significantly reduced ALT levels and a trend toward lower AST levels (P = 0.08; Fig. 5A). In contrast, CCR5−/− showed no significant reduction in the liver damage induced by 4-1BB. Ablation of these chemokine receptors...
receptors individually failed to impact the ability of 4-1BB agonist therapy to mediate rejection of subcutaneous melanoma (Fig. 5B), implying either that they are not required, or that sufficient redundancy exists to preserve responses in the tumor setting. Moreover, removing these chemokine receptor pathways did not significantly affect recruitment of antigen-specific T cells into the tumor (Fig. 5C). Of note, the apparent lack of significant increase in tetramer frequency in response to 4-1BB therapy here is largely a function of the potency of 4-1BB agonists against these B16-Ova tumors. In the treated animals, both WT and chemokine knockout, the therapy is so effective that a significant number of mice have eradicated their tumors, leaving only a small remnant of Matrigel and few, if any, antigen-specific CD8 T cells. It has been demonstrated across multiple tumor microenvironments that increased CD8/Treg ratios correlate with more successful responses to immune-based therapies (12, 38, 39). We found that the magnitude of elevation of CD8/Treg ratios in WT, CCR2<sup>−/−</sup>/C<sup>0</sup>/C<sup>0</sup>, CXCR3<sup>−/−</sup>/C<sup>0</sup>/C<sup>0</sup>, and CCR5<sup>−/−</sup>/C<sup>0</sup>/C<sup>0</sup> mice were not significantly different, providing additional evidence that loss of a single chemokine receptor pathway does not impact antitumor immune responses (Fig. 5D; Supplementary Fig. S5D). Interestingly, within the liver, abrogation of CCR5 significantly increased the CD8/Treg ratio. While this may be beneficial in the tumor setting, an increased ratio within the liver may account for the maintenance of elevated transaminase elevation in the CCR5 knockout mice.

Figure 4. Tregs suppress 4-1BB agonist antibody–induced liver pathology. A, WT mice or mice lacking MHC class I expression (β2M<sup>−/−</sup>) or all MHC class II alleles (MHC-II<sup>−/−</sup>) were treated for three rounds with 4-1BB agonist antibody (days 0, 3, and 6) before mice were bled for serum liver enzyme analysis 16 days after beginning treatment. Serum ALT and AST were measured upon sacrifice as units of enzyme/liter of blood. B, Mice were sublethally irradiated (500 rad) before administration of 2 × 10<sup>6</sup> CD8 splenocytes. WT mice or β2M<sup>−/−</sup> mice received splenocytes from WT mice (WT CD8—WT) or (WT CD8—β2M<sup>−/−</sup>), respectively. Mice were subsequently treated with three rounds of 4-1BB immunotherapy. Treated mice were then bled 16 days after first administration of therapy, and serum ALT and AST were measured. C, Frequency of Treg infiltration into the perfused livers of treated mice 16 days after initiation of therapy was quantified by flow cytometric analysis as the percentage of Foxp3<sup>+</sup>CD4<sup>+</sup> cells as a fraction of total CD4<sup>+</sup> T cells. D, Mice received 5 × 10<sup>5</sup> CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> splenocytes FACS-sorted from naïve mice 1 day prior to treatment. Concurrently, mice expressing the DT receptor under control of the Foxp3 promoter (Foxp3-DTR) were administered 10 µg/kg body weight of DT 1 day prior to initiation of therapy and every 3 days thereafter until completion of the experiment. Data were pooled from ≥2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student t test applying Welch correction for unequal variance. ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001.
Figure 5.
The chemokine receptors CCR2 and CXCR3 contribute to 4-1BB agonist-induced liver pathology. WT mice or mice lacking specific chemokine receptors (CCR2−/−, CXCR3−/−, or CCR5−/−) were subcutaneously implanted on the right flank with 3 × 10^5 B16 melanoma tumor cells expressing the ovalbumin antigen (B16-Ova). At 3-day intervals after initial tumor challenge (days 3, 6, and 9), mice were treated with antibody immunotherapy delivered i.p. in combination with an irraditated tumor vaccine (FVAX) administered subcutaneously on the left flank. Mice were bled for serum liver enzyme analysis 16 days after treatment initiation. Mice were then sacrificed, and perfused livers and tumors were extracted, weighed, and processed for FACS analysis. A, Serum ALT and AST were measured upon sacrifice as units of enzyme/liter of blood volume. B, Upon sacrifice, tumors were harvested and weighed. C, Tumor infiltration of Ova-specific CD8+ T cells was determined by staining tumor-infiltrating lymphocytes (TIL) with fluorocently labeled Ova257-254/Kb (SIINFEKL) tetramer and antibodies to CD8. Data are expressed as the total number of tetramer positive cells per milligram of tumor. D, Quantification of CD8/Treg ratios within the tumor and liver was performed by dividing the number of CD8+CD3+ cells by the number of CD4+Foxp3+ cells found within the tissue infiltrate. Data were pooled from ≥2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student t test applying Welch correction for unequal variance. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
(Fig. 5A). The lack of an increase in transaminases in these CCR5 knockout mice, we hypothesize, suggests that Treg may rely on production of soluble factors such as TGF-β, rather than on cell-contact dependent interactions to maintain liver homeostasis, and therefore can maintain tissue tolerance even when at a modest numerical disadvantage relative to effectors.

Taken together, these data suggest that immune infiltration into the liver and tumor can be uncoupled through abrogation of chemokine receptor signaling. Further, CCR2 and CXCR3 appear to be critical mediators of α4-1BB–induced hepatotoxicity-mediated T-cell trafficking, while disengaging these pathways does not significantly impact the ability of α4-1BB therapy to generate potent antitumor immunity.

Discussion

While the field of immunotherapy has experienced unprecedented growth due to the success of immune checkpoint blockade, clinical translation of the most efficacious monoclonal antibodies is hindered by immune toxicities. 4-1BB agonist antibodies are among the most effective immunotherapeutics across preclinical models of cancer (4). Severe off-target liver damage in early phase I trials, however, has limited the clinical progression of highly active 4-1BB antibodies (10). Effective prophylaxis, biomarker prediction, or management of this toxicity, except through highly attenuated dosing, has proven challenging due to lack of mechanistic understanding of the underlying cellular and molecular mechanisms. Efforts at development of 4-1BB agonist antibodies with limited toxicity are ongoing; however, no 4-1BB agonist has advanced beyond early phase II trials. In this article, we sought to uncover the mechanisms driving 4-1BB agonist–mediated liver pathology so that this knowledge may inform both antibody engineering and combination 4-1BB agonist trial design.

The capacity of 4-1BB activation to potentiate CD8 T cell responses is widely accepted; however, we find that activation of liver myeloid cells, not T cells, is a critical initiating step that triggers hepatotoxicity. Following α4-1BB administration, bone marrow–derived monocytes infiltrate the liver and, in response to 4-1BB activation, initiate a cascade of inflammatory cytokine production that triggers 4-1BB upregulation by resident Kupffer cells, allowing these cells to subsequently respond to agonist antibody. Antigen presentation capacity increased in multiple Kupffer cell populations based on MHC-II upregulation. In addition, the cytokine-producing CD11b+ subsets increased production of IL27 more than 20-fold. We find that this augmented IL27 production is essential for the progression of liver inflammation, as neither EBI3−/− nor IL27Ra−/− mice showed any evidence of transaminase elevation in response to 4-1BB activation. Despite the requirement for myeloid initiation, CD8 T cells mediate the actual liver injury, as mice lacking CD8s fail to develop transaminase elevation. Prior studies indicate that mice expressing only CD8 T cells specific for an Ovalbumin-peptide/H2-Kb complex were also resistant to α4-1BB liver toxicity (9). This observation, coupled with our own B2M−/− data, led us to question whether CD8 T-cell activation downstream of myeloid 4-1BB activation was occurring via an antigen-dependent or independent mechanism. Mice with WT CD8 T cells that were incapable of MHC class I antigen presentation failed to develop liver injury in response to α4-1BB, suggesting that hepatotoxic CD8 T cells recognize uncharacterized liver-specific auto-antigens. It is likely, then, that 4-1BB activation of myeloid cells leads to enhanced presentation of liver tissue antigens and secreted IL27 further provides a critical signal 3 for liver autoreactive CD8 T cell activation. The role of IL27, in this context, could be direct costimulation of effector CD8 and/or inhibition of Treg suppressive activity. These mechanistic insights suggest IL27 blockade as a means to reduce 4-1BB agonist liver toxicity; however, we have previously found IL27 to play a critical role in effector T cell polarization downstream of α4-1BB as well as in antitumor responses (14, 40, 41). Currently, the only described mechanism to reduce 4-1BB agonist liver toxicity involves combination therapy with CTLA-4 blockade (8). We confirm the capacity of this combination to block 4-1BB agonist transaminase elevation. Given this combination also shows therapeutic synergy and the capacity to limit αCTLA-4 IA/E (8, 12), it remains unfortunate that no trials have tested α4-1BB/αCTLA-4 in patients. In contrast, the α4-1BB/αPD-1 combination has been tested in patients, but with very limited dosing regimens due to the capacity of αPD-1 to worsen α4-1BB–mediated hepatitis—an effect we also validated herein (17). We hypothesized that the liver-protective effect of CTLA-4 blockade might also extend to α4-1BB/αPD-1 combination therapy; however, the effect of PD-1 blockade was, in fact, dominant and that triple combination treatment engendered severe transaminitis. Differential effects of CTLA-4 and PD-1 checkpoint blockade on α4-1BB–mediated liver toxicity may be due, in part, to the expression patterns of each receptor on distinct immune populations (high CTLA-4, moderate PD-1: Tregs, low CTLA-4, high PD-1; CD8) or on potential potency of these receptors to inhibit T-cell activation/effectector responses. Alternatively, PD-1 blockade may decrease the suppressive capacity of Treg, and our data suggest that CTLA-4 blockade requires the presence of (functional) Treg to ameliorate 4-1BB agonist liver toxicity (42). In the context of our model (Fig. 6), CTLA-4 blockade limited the accumulation of T cells in the liver following 4-1BB agonist administration and thus attenuated resulting hepatotoxicity. While we were unable to identify a distinct cellular mechanism underlying this effect, we were able to demonstrate an impact of αCTLA-4 coadministration on myeloid infiltration and effector function in the liver; however, this mechanism may also involve a qualitative change in Treg phenotype (no quantitative changes were detected). We observed distinct patterns of parenchymal versus perivascular infiltration of F4/80+ cells in each combination setting. We hypothesize that it is the combination of accumulation of F4/80+ cells in the perivascular area coupled with a capacity to infiltrate the parenchyma, which equals or exceeds that of 4-1BB agonist alone, that explains why the triple combination induces exacerbated liver toxicity. Although perivascular infiltration increases with the αCTLA-4/α4-1BB combination, parenchymal F4/80+ cell density decreases coincident with a decrease in CD8 T cells in this region. Liver damage associated with significant transaminase elevation, in general, requires infiltration and damage within the liver parenchyma itself. Perivascular accumulation can represent expansion of resident cells with progenitor capacity and/or infiltration of monocytes and their subsequent differentiation into F4/80+ cells (a phenomenon for which we have demonstrated a limited capacity).
We next considered whether the chemokine receptors governing entry of hepatitis-inducing T cells into the liver, versus migration of tumor-specific T cells into melanoma tumors might be sufficiently different to separate tumor immunity from hepatotoxicity. We found that CCR2/−/− mice, and to a lesser extent CXCR3/−/− mice, were protected from 4-1BB agonist-induced liver toxicity but were still capable of effectively combating B16-Ova tumors growing on the flank. The impact of CCR2 knockout in abrogating liver toxicity remains enticing, as both small molecule (CCX872, ChemoCentryx; PF-04136309, Pfizer) and antibody (MLN1202, Millennium) antagonists for CCR2 are currently in clinical trials. Given our findings, 4-1BB agonist antibodies administered in combination with CCR2 inhibitors may prove to be a potent combination in promoting tumor regression while inhibiting off-target liver toxicity.

Disclosure of Potential Conflicts of Interest
D.S. Hong reports receiving commercial research grants from Pfizer. M.A. Curran is a consultant/advisory board member for Compass Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Figure 6.
Mechanistic model of 4-1BB agonist antibody-mediated hepatotoxicity.
Activation of 4-1BB on Liver Myeloid Cells Triggers Hepatitis via an Interleukin-27–Dependent Pathway

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