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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 co-stimulatory receptor 4–1BB (CD137) are among the most effective immunotherapeutic agents across pre-clinical 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 co-administration of checkpoint blockade, via 1) measurement of serum transaminase levels, 2) imaging of liver immune infiltrates, and 3) 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. Co-administration of CTLA-4 blockade ameliorates transaminase elevation, whereas PD-1 blockade exacerbates it. Loss of the

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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. Co-administration of CTLA-4 and/or CCR2 blockade may minimize hepatitis, but yield equal or greater antitumor immunity.

Keywords

4-1BB; immunotherapy; Interleukin-27; CCR2; CTLA-4

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 co-inhibitory receptors, agonist antibodies which activate tumor necrosis factor superfamily receptors have demonstrated significant therapeutic potential both in pre-clinical models and clinical trials (3). Among these agonists, activators of the co-stimulatory receptor 4–1BB (CD137) have demonstrated exceptional potency across multiple pre-clinical 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 α 4–1BB antibody therapy promotes liver damage, and to explore potential avenues to uncouple augmentation of anti-tumor 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 (IL-27), 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 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 α 4–1BB-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 hepatogenic immune responses following 4–1BB agonist therapy rely heavily on the

chemokine receptors CCR2 and, less so, to CXCR3, these receptors appear to be largely dispensable for anti-melanoma 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 (6wk) C57BL/6 mice were purchased from Taconic Biosciences (Hudson, NY). *4-1BB*^{-/-}, *EBI3*^{-/-}, *IL27 receptor alpha*^{-/-}, *β2M*^{-/-}, *MHCII*^{-/-}, *Foxp3-DTR*, *CXCR3*^{-/-}, *CCR2*^{-/-}, and *CCR5*^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All procedures were conducted in accordance with the guidelines established by the U.T. MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Cell lines and reagents

B16 melanoma, B16-Flt3-ligand (FVAX) and B16-Ova were obtained/created and cultured as described (11,12).

The BV421-labeled H2-K^b epitope OVA²⁵⁷⁻²⁶⁴ (SIINFEKL)-containing tetramer was acquired from the Tetramer Core Facility at the National Institute of Health (Emory University, Atlanta GA).

Therapeutic antibodies

T cell co-stimulatory 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 urelumab 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.

Immune ablation and reconstitution

C57BL/6 mice or 4-1BB^{-/-} mice were sub-lethally irradiated (500 rads) using a Cesium-137 irradiator. One day later, splenic lymphocytes were isolated using CD90.2 magnetic beads (Miltenyi Biotec, San Diego, CA) and injected i.v. at 2×10⁶ cells/mouse into irradiated hosts.

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, *CCR2*^{-/-}, *CXCR3*^{-/-}, or *CCR5*^{-/-} mice were implanted s.c. with 3×10^5 B16-Ova cells on the flank as described (11,12). On days 3,6, and 9 mice received $\alpha 4$ -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 μ g/kg one day prior to $\alpha 4$ -1BB and every 3 days thereafter until sacrifice. Alternately, CD4⁺CD25⁺CD3⁺ cells were FACS sorted from naïve spleens and 5×10^5 cells were injected into host mice one day prior to immunotherapy.

Myeloid cells were adoptively transferred by magnetically sorting bone marrow-derived monocytes using a monocyte isolation kit (Miltenyi Biotec, Auburn CA). Sorted cells (CD45.2) were adoptively transferred at 2×10^6 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 antibodies at a time from Biolegend, BD Biosciences, and Thermo. Flow cytometry data was 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 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 semi-quantitative 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, were counted separately in a microscopic field at 20X 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 et.al. 2015.

Immunofluorescence staining and imaging

Tissues were collected and flash frozen in liquid nitrogen. The frozen tissues were embedded in Tissue-Tek® OCT Compound (Sakura, Torrance, CA) 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, Carlsbad, CA). Confocal imaging was performed using a TCS SP8 laser-scanning confocal microscope equipped a 20X objective (HCPL APO 20X/0.70 NA), Leica Microsystems) with lasers for excitation at 405nm, 458nm, 488nm, 514nm, 543nm, and 633nm wavelengths. (Leica Microsystems, Inc., Bannockburn, IL).

Real Time PCR

Liver myeloid subpopulations were sorted as shown (Supplemental Fig. 1) at the MD Anderson Flow Cytometry and Cellular Imaging Core Facility (FCCIF). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, MD) and reverse transcribed using the SuperScript IV Reverse Transcriptase kit (Thermo). Taqman real-time PCR was performed on a Via 7 Real Time PCR System (Applied Biosystem, CA) as previously described (13,14). Levels of *il27-p28*, *ifng*, and *tnfa* were expressed as the fold change using the Ct method.

Cytometric Bead Array

Bone marrow derived monocytes were isolated from wildtype mice using a Monocyte Isolation Kit (Miltenyi Biotech) and were stimulated *in vitro* with α4-1BB (3H3) antibody for 48 hours. Cytokine release was quantified using a Th1/Th2/Th17 Cytometric Bead Array kit (BD) as per 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's T test applying Welch's correction for unequal variance. Graphs show mean ± standard deviation unless otherwise indicated. P-values less than 0.05 were considered 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 α4-1BB agonist antibody induced liver pathology, mice

were treated with three administrations of checkpoint antibody, α 4-1BB alone, α 4-1BB in combination with α CTLA-4 or α PD-1, or triple combination therapy. At the peak of hepatic injury, sixteen days after the initiation of treatment (Supplemental Fig. 1A), 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) (15). As noted previously, co-administration of α CTLA-4 significantly decreased serum transaminase levels compared to α 4-1BB monotherapy (8), whereas dual therapy with α 4-1BB and α PD-1 significantly increased transaminase levels (Fig. 1A) (16). The protective effect of α CTLA-4 therapy was lost when given in combination with both α 4-1BB and α PD-1, suggesting that exacerbation of hepatitis by α PD-1 dominates over the capacity of α CTLA-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 α 4-1BB hepatotoxicity.

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, Supplemental Fig. 1B), but did not significantly impact infiltration of bulk CD4⁺ T cells (CD4⁺CD3⁺) or CD4⁺ effector T cells (CD4⁺CD3⁺FoxP3⁻) (Supplemental Fig. 2A, and B). 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 anti-tumor immunity by exhibiting elevated cytotoxicity compared to their Th1/Tc1 counterparts, and likely play a significant role in mediating liver damage (Supplemental Fig. 2 C,D,E)(14,17–19). Further, the addition of CTLA-4 blockade to α 4-1BB treatment reduced the frequency of T cell infiltration into the liver versus α 4-1BB alone (Fig. 1B). Whereas the overall CD3 density was reduced in α 4-1BB/ α CTLA-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, Supplemental Fig. 2D,E). 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 α CTLA-4 was co-administered with α 4-1BB, but were exacerbated by triple combination therapy (Fig. 1D, E). Overall, α CTLA-4 co-administration with α 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,F).

To test whether the ability of CTLA-4 blockade to reduce liver pathology was specific for 4-1BB agonist therapy, we also tested α CTLA-4 in combination with antibodies targeting the TNF receptor CD40. Co-stimulation through CD40 induces an acute and transient hepatic injury that peaks within a week of antibody administration and declines thereafter, whereas 4-1BB agonists induced 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 α 4-1BB, α CD40-induced liver damage was not ameliorated by co-administration with α CTLA-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 α CD40 or α 4-1BB/ α PD-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 (20), 4-1BB can be expressed on both T cell, NK cell and myeloid populations (4,14,21,22), and the relative contribution of each of these to liver pathology remains undefined.

To reveal the relative contribution of the myeloid versus lymphocyte compartments to α 4-1BB induced hepatotoxicity, wildtype 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 wildtype or 4-1BB^{-/-} mice were magnetically sorted and adoptively transferred into irradiated wildtype or 4-1BB^{-/-} hosts. In this way, ablation of the lymphoid pool, but not the radio-resistant 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 to WT to WT transfers administered isotype control antibodies or 4-1BB^{-/-} mice receiving 4-1BB^{-/-} cells in conjunction with α 4-1BB (Fig. 2A), while AST elevation, which is always less affected by α 4-1BB, showed modest elevation as well (Supplemental Fig. 3A). Wildtype 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 wildtype 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, α 4-1BB could no longer trigger hepatotoxicity suggesting a requirement for myeloid 4-1BB activation to initiate a liver 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 α 4-1BB induced liver pathology. We found that, in comparison to untreated livers, α 4-1BB therapy increased the frequency of F4/80⁺ macrophages within the liver parenchyma which was significantly reduced by combining α CTLA-4 with α 4-1BB (Fig. 2B, C, D). Interestingly, combination therapy favored accumulation of F4/80⁺ cells within the perivascular space compared to 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 (23). Further, Kupffer cells can be sub-classified 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 (24). In naïve mice, we were only able to detect clear 4-1BB expression on monocytes by flow cytometry (Supplemental Fig. 3B); however, 4-1BB expression was detected on both F4/80⁻ monocytes and on a small percentage of F4/80⁺ Kupffer cells *in situ* by immuno-fluorescence (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 TNF α which are plentiful during α 4-1BB-induced liver injury, with flow cytometry confirming the CD11b⁻CD68⁺ and CD11b⁺CD68⁺ sub-populations as the primary targets (Fig. 2E, Supplemental Figure 3C). To assess the origin of these Kupffer cell populations, as well as the plasticity of infiltrating bone marrow-derived monocytes, we adoptively transferred congenically labelled bone marrow myeloid progenitor cells and administered α 4-1BB to the recipient mice. In response to 4-1BB activation, these monocytes expanded in the blood and infiltrated the liver (Supplemental Fig. 3D). 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 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(25,26).

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 (Supplemental Figure 3E) which triggers 4-1BB upregulation by resident Kupffer cells allowing them to respond in turn to the agonist antibody(Supplemental Figure 3C). Our data, however, does 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 suggests that these cells are either new emigrants or derived from them, or, alternatively that they are re-distributing within sub-compartments of the liver. Both possibilities are consistent with increased infiltration into the perivascular space that we observed (27,28). 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 published literature demonstrating that this activation promotes enhanced co-stimulatory capacity (14,21).

We next sought to confirm the ability of the cytokine-producing myeloid populations to mediate liver damage during the course of α 4-1BB 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 (Supplemental Fig. 1), and RNA was isolated from each population for quantitative real time PCR. We found that, compared to α CD40 treatment which induced significant activation and IFN γ 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 α 4-1BB treated mice. Within the two CD11b⁺CD68⁻ subsets, we observed approximately 20-fold increased expression of *IL-27-p28* following 4-1BB agonist therapy compared to 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 TNF α . Notably, the cytokine producing subsets of myeloid cells produced less IL-27 and TNF α in mice receiving the α 4-1BB/ α CTLA-4 combination therapy compared to mice receiving α 4-1BB monotherapy. While the CD11b⁻CD68⁺ subset demonstrated roughly 50-fold increases in IL27-p28 expression relative to its baseline level during α 4-1BB/ α CTLA-4 combination therapy, the delayed cycle within which transcripts were detected (~cycle 37 versus 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, α 4-1BB-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 IL-27. Co-administration of CTLA-4 blockade reduced inflammatory cytokine production in these subsets, consistent with the reduced transaminase elevation observed in those mice.

Interleukin 27 is a critical regulator of liver inflammation.

In addition to the above, we previously reported that IL-27 acts to polarize T cells to the cytotoxic ThEO/TcEO phenotype (14), and therefore hypothesized that it may play a role in triggering α 4-1BB-induced hepatic damage. To evaluate the contribution of IL-27 to immune-mediated hepatotoxicity, mice lacking the Ebi3 subunit of IL-27 (*EBI3*^{-/-}) or mice lacking the IL-27 receptor alpha subunit (*IL27Ra*^{-/-}) were treated with α 4-1BB therapy followed by analysis of transaminase levels. Compared to wildtype 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 α 4-1BB/ α CTLA-4/ α PD-1 therapy was also nearly completely abrogated in *EBI3*^{-/-} mice. Moreover, abrogation of the IL-27 pathway did not significantly impact basal 4-1BB expression nor TNF α induced expression on liver-resident myeloid populations (Supplemental Fig. 4A, B), suggesting that *EBI3*^{-/-} mice were equally capable of receiving 4-1BB signal.

In mice lacking the IL-27/IL-27R 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 IL-27 pathway (Supplemental Fig. 4C). While the percent of CD4⁺Eomes⁺KLRG1⁺ ThEO phenotype cells (Supplemental Fig. 4D), and CD8⁺ TcEO phenotype T cells were minimally affected by loss of IL-27, the total numbers of the highly inflammatory TcEO population within liver infiltrates were significantly diminished absent functional IL-27 signaling (Fig. 3D).

Taken together, these data demonstrate a critical requirement for the inflammatory cytokine IL-27 in mediating 4-1BB agonist antibody-induced hepatotoxicity as well as for recruitment and/or expansion of hepatogenic T cells into the liver.

Regulatory T cells restrict 4-1BB agonist antibody induced liver pathology

Given the ability of myeloid cells to activate T cell responses, coupled with the capacity of IL-27 to act as an inflammatory mediator of hepatic damage with pleiotropic effects on helper T cell polarization, Treg suppression, and T cell trafficking (29–31), and the prolonged inflammatory response induced by α 4-1BB (Fig. 1G), we investigated the role of T cells in propagating α 4-1BB-mediated liver damage. To assess the relative contribution of the T cell pool in mediating hepatotoxicity, we administered α 4-1BB to mice lacking the β 2 microglobulin subunit of the major histocompatibility (MHC) I complex (β 2M^{-/-}) 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 to wildtype mice (Supplemental Fig. 5A,B), elevation of liver ALT and AST levels was completely abrogated in α 4-1BB-treated β 2M^{-/-} mice, confirming the role of CD8⁺ T cells in mediating the bulk of the liver damage (Fig. 4A) (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 sub-lethally irradiated and CD8⁺ splenocytes from wildtype mice were transferred into β 2M^{-/-} mice. We hypothesized that if the lack of CD8 T cells in these mice was the sole cause of the abrogated hepatotoxicity, then supplying wildtype CD8⁺ T cells would reinitiate toxicity. Interestingly, supplementation of WT CD8⁺ T cells into β 2M^{-/-} 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 to α 4-1BB treated wildtype mice (Fig. 4A).

We next hypothesized that exacerbation of hepatotoxicity in *MHCII*^{-/-} mice stemmed, not from dysregulation of effector T cells responses, but from elimination of Treg cells 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⁺ regulatory T cells in the livers of α 4-1BB compared to untreated mice (Fig. 4C) suggesting that Treg expansion might be acting to

limit hepatitis. To validate a role for Tregs in limiting $\alpha 4$ –1BB-induced liver toxicity, we treated mice expressing the diphtheria toxin (DT) receptor (DTR) under control of the Foxp3 promoter (Foxp3-DTR) in which Foxp3⁺ regulatory T cells can be depleted upon administration of DT. Briefly, DT was administered 2 days before $\alpha 4$ –1BB therapy, and continued until the end of treatment for complete and sustained Treg depletion. Treg depletion was successful based on analysis of blood three days before serum analysis (Supplemental Fig. 5C). Consistent with our hypothesis, depletion of Tregs significantly aggravated $\alpha 4$ –1BB induced liver damage, increasing AST and ALT levels 5–6-fold, and eliminating the ability of α CTLA-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 Treg cells are critical suppressors of inflammation during $\alpha 4$ –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 Fc γ RIV receptor in these tissues(32).

Taken together this data suggests a critical role of CD8 T cell activation in mediating $\alpha 4$ –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 $\alpha 4$ –1BB.

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

Given the ability of IL-27 to induce chemokine receptor expression (33,34), the reduced immune infiltrate in the liver in the absence of IL-27, and the reduced myeloid presence in mice treated with $\alpha 4$ –1BB/ α CTLA-4 co-therapy, 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 (28,35), we sought to determine whether anti-tumor immunity could be separated from hepatitis based on differential homing. We challenged either wildtype, CCR2^{-/-}, CXCR3^{-/-}, or CCR5^{-/-} mice subcutaneously with 3 $\times$ 10⁵ 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 IFN γ -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 (36). 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 towards lower AST levels (p=0.08) (Fig. 5A). In contrast, CCR5^{-/-} showed no significant reduction in the liver damage induced by $\alpha 4$ –1BB. Ablation of these chemokine 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 $\alpha 4$ -1BB therapy here is largely a function of the potency of 4-1BB agonists against these B16-Ova tumors. In the treated animals, both wild-type 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 (12,37,38). We found that the magnitude of elevation of CD8/Treg ratios in wildtype, CCR2^{-/-}, CXCR3^{-/-}, and CCR5^{-/-} mice were not significantly different providing additional evidence that loss of a single chemokine receptor pathway does not impact anti-tumor immune responses (Fig. 5D, Supplemental Fig 5D). 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 (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 $\alpha 4$ -1BB induced hepatotoxicity-mediated T cell trafficking, while disengaging these pathways does not significantly impact the ability of $\alpha 4$ -1BB therapy to generate potent anti-tumor 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 mono- and combination therapies from pre-clinical models has been limited by immune toxicities. 4-1BB agonist antibodies are among the most effective immunotherapeutics across pre-clinical 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 manuscript, 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 $\alpha 4$ -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 IL-27 more than 20-fold. We find that this augmented IL-27 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 β2M^{-/-} 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 wildtype 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 IL-27 further provides a critical signal 3 for liver auto-reactive CD8 T cell activation. The role of IL-27, in this context, could be direct co-stimulation of effector CD8 and/or inhibition of Treg suppressive activity. These mechanistic insights suggest IL-27 blockade as a means to reduce to 4-1BB agonist liver toxicity; however, we have previously found IL-27 to play a critical role in effector T cell polarization downstream of α4-1BB as well as in anti-tumor responses (14,39,40).

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 IRAE (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 (16). 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/effector responses. Alternatively, PD-1 blockade may decrease the suppressive capacity of Treg, and our data suggests that CTLA-4 blockade requires the presence of (functional) Treg to ameliorate 4-1BB agonist liver toxicity(41). 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 co-administration 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

4-1BB (CD137) agonist antibodies are among the most potent immunotherapeutic agents across a broad spectrum of pre-clinical 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 co-stimulation of cytotoxic CD8 T cells which mediate liver tissue injury and transaminase elevation. We show that co-blockade of CTLA-4 and/or CCR2 can ameliorate hepatitis without impairing tumor immunity providing a path to clinical translation.

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 alanine aminotransferase (ALT) and aspartate aminotransferase (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. Percent of CD3⁺ cells was calculated as a fraction of liver CD45⁺ cells. Frequency of CD8⁺ T cells was calculated as a percent of CD3⁺ cells. **C**) Hematoxylin and Eosin (H&E) staining or immunohistochemistry (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 α CTLA-4 blockade. Mice were then bled at the peak of α CD40-mediated liver damage (D8) 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 20X magnification. Data were pooled from 3 experiments with 5 mice per group. Bars represent mean $\pm$ SD. Statistical significance was calculated using a two-sided Student's T test applying Welch's correction for unequal variance. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 2: Administration of 4-1BB agonist antibodies initiates liver pathology through activation of liver-resident myeloid cells.

A) Mice were sublethally irradiated (500 rads) before administration of 2×10^6 CD90⁺ splenocytes. Wildtype mice either received splenocytes from wildtype mice (WT→WT) or from 4-1BB^{-/-} mice (4-1BB^{-/-}→WT) and 4-1BB^{-/-} mice received splenocytes from wildtype mice (WT→4-1BB^{-/-}) or from 4-1BB^{-/-} mice (4-1BB^{-/-}→4-1BB^{-/-}). Mice were subsequently treated with three round of isotype control or α4-1BB 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 wildtype mice administered either α4-1BB therapy alone or in combination with αCTLA-4 checkpoint blockade. Myeloid infiltration shown as the percent of F4/80⁺ cells as a fraction of total CD45⁺ cells. **C)** Immunohistochemistry staining for F4/80⁺ was performed on sectioned 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 naïve or α4-1BB-treated livers 16 days after initiation of treatment **F)** Phenotypic characterization of

congenically marked, adoptively transferred bone marrow-derived myeloid cells into perfused livers and blood based on flow cytometry of mice administered $\alpha 4-1\text{BB}$ therapy. **G)** Frequency of inflammatory/activation markers based on flow cytometry of perfused livers from treated mice based on three subsets of liver-resident macrophages: $\text{CD11b}^+\text{CD68}^-$ cytokine-producing Kupffer cells, $\text{CD11b}^+\text{CD68}^+$ cytokine-producing/phagocytic Kupffer cells, and $\text{CD11b}^-\text{CD68}^+$ phagocytic Kupffer cells. **H)** Gene expression from individual myeloid populations was calculated at day 7 post 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 20X magnification. Micrographs in E were imaged using a 20X air objective. Insets for magnified using 2X magnification. Gene expression was calculated using Taqman primers via the Ct method. Data were pooled from 2 experiments with 5 mice per group. Bars represent mean $\pm$ SEM. Statistical significance was calculated using a two-sided Student's T test applying Welch's correction for unequal variance. ns, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Figure 3: Interleukin 27 is a critical regulator of 4-1BB agonist-induced liver inflammation. Wildtype mice or mice lacking the Ebi3 subunit of the IL-27 cytokine complex (EBI3^{-/-}) or the IL-27 receptor alpha subunit (IL27Ra^{-/-}) were treated for three rounds of $\alpha 4-1BB$ agonist immunotherapy before analysis of serum transaminase levels and hepatic immune infiltrates 16 days after initiation of treatment. **A)** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured upon sacrifice as units of enzyme/liter of blood volume. **B)** Quantification of immune infiltrates within perfused livers of treated mice was measured by flow cytometry. Frequency of CD3⁺ cells was calculated as a percent of total CD45⁺ cells in the liver. **C)** Frequency of CD8⁺ T cells was calculated as a percent of CD3⁺ cells. Total numbers of cells were taken as number of CD3⁺ or CD3⁺CD8⁺ cells within perfused livers. **D)** Quantification of percent and total numbers of TcEO T cell infiltration within the livers of treated mice. Frequency of TcEO was calculated based on the percent 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 $\pm$ SD. Statistical significance was calculated using a two-sided Student's T test applying Welch's correction for unequal variance. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 4: Regulatory T cells suppress 4-1BB agonist antibody induced liver pathology. **A)** Wildtype mice or mice lacking MHC Class I expression ($\beta 2M^{-/-}$) or all MHC Class II alleles (MHC-II $^{-/-}$) were treated for three rounds with $\alpha 4-1BB$ 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 sub-lethally irradiated (500 rads) before administration of 2×10^6 CD8 $^{+}$ splenocytes. Wildtype mice or $\beta 2M^{-/-}$ mice received splenocytes from wildtype mice (WT CD8 $\rightarrow$ WT) or (WT CD8 $\rightarrow$ $\beta 2M^{-/-}$) respectively. Mice were subsequently treated with three round of $\alpha 4-1BB$ immunotherapy. Treated mice were then bled 16 days after first administration of therapy and serum ALT and AST were measured. **C)** Frequency of regulatory T cell (Treg) infiltration into the perfused livers of treated mice 16 days after initiation of therapy was quantified by flow cytometric analysis as the percent of Foxp3 $^{+}$ CD4 $^{+}$ cells as a fraction of total CD4 $^{+}$ T cells. **D)** Mice received 5×10^5 CD3 $^{+}$ CD4 $^{+}$ CD25 $^{+}$ splenocytes FACS-sorted from naïve mice one day prior to treatment. Concurrently, mice expressing the diphtheria toxin receptor under control of the Foxp3

promoter (Foxp3-DTR) were administered 10 µg/kg body weight of diphtheria toxin one day prior to initiation of therapy and every three 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's T test applying Welch's correction for unequal variance. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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Figure 5: The chemokine receptors CCR2 and CXCR3 contribute to 4-1BB agonist-induced liver pathology.

Wildtype 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 three-day intervals after initial tumor challenge (days 3, 6, and 9) mice were treated with antibody immunotherapy delivered i.p. in combination with an irradiated 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 fluorescently labeled Ova^{257–254}/K^b (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 were calculated 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's T test applying Welch's correction for unequal variance. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 6: Mechanistic model of 4-1BB agonist antibody-mediated hepatotoxicity.