Dual Role of B7 Costimulation in Obesity-Related Non-alcoholic Steatohepatitis and Metabolic Dysregulation

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The low-grade inflammatory state present in obesity contributes to obesity-related metabolic dysregulation, including non-alcoholic steatohepatitis (NASH) and insulin resistance. Intercellular interactions between immune cells or between immune cells and hepatic parenchymal cells contribute to the exacerbation of liver inflammation and steatosis in obesity. The costimulatory molecules, B7.1 and B7.2, are important regulators of cell-cell interactions in several immune processes; however, the role of B7 costimulation in obesity-related liver inflammation is unknown. Here, diet-induced obesity (DIO) studies in mice with genetic inactivation of both B7.1 and B7.2 (double knockout; DKO) revealed aggravated obesity-related metabolic dysregulation, reduced insulin signalling in the liver and adipose tissue (AT), glucose intolerance, and enhanced progression to steatohepatitis resulting from B7.1/B7.2 double deficiency. The metabolic phenotype of B7.1/B7.2 double deficiency upon DIO was accompanied by increased hepatic and AT inflammation, associated with largely reduced numbers of regulatory T cells (Tregs) in these organs. In order to assess the role of B7 costimulation in DIO in a non-Treg-lacking environment, we performed antibody (Ab)-mediated inhibition of B7 molecules in wild-type mice in DIO. Antibody-blockade of both B7.1 and B7.2 improved the metabolic phenotype of DIO mice, which was linked to amelioration of hepatic steatosis and reduced inflammation in liver and AT. Conclusion: Our study demonstrates a dual role of B7 costimulation in the course of obesity-related sequelae, particularly NASH. The genetic inactivation of B7.1/B7.2 deteriorates obesity-related liver steatosis and metabolic dysregulation, likely a result of the intrinsic absence of Tregs in these mice, rendering DKO mice a novel murine model of NASH. In contrast, inhibition of B7 costimulation under conditions where Tregs are present may provide a novel therapeutic approach for obesity-related metabolic dysregulation and, especially, NASH. (HEPATOLOGY 2014;60:1196-1210)

Obesity is associated with low-grade inflammation, liable for the development of insulin resistance (IR), type 2 diabetes, and non-alcoholic steatohepatitis (NASH). Immune cells of both innate and adaptive immunity are implicated in this process and contribute to the development of

Abbreviations: Ab, antibody; AKT, protein kinase B; ALT, alanine transaminase; APCs, antigen-presenting cells; AST, aspartate transaminase; AT, adipose tissue; ChREBP, carbohydrate-responsive element-binding protein; CTLA-4, cytotoxic T lymphocyte-associated molecule 4; DCs, dendritic cells; DIO, diet-induced obesity; DKO, double knockout; FAS, fatty acid synthase; FCM, flow cytometry; Foxp3, forkhead box protein 3; GLDH, glutamate dehydrogenase; GLUT, glucose transporter; GTT, glucose tolerance test; HFD, high-fat diet; HSC, hepatic stellate cell; IL, interleukin; IR, insulin resistance; KCs, Kupffer cells; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; ND, normal diet; NPC, nonparenchymal cell; PCNA, proliferating cell nuclear antigen; PGC, peroxisome proliferator-activated receptor-gamma coactivator; qPCR, quantitative polymerase chain reaction; RER, respiratory exchange ratio; SC, subcutaneous; SREBP-1C, sterol regulatory element-binding protein 1C; SVE, stromal vascular fraction; TG, triglyceride; TGF-β, transforming growth factor beta; TNE, tumor necrosis factor; Tregs, regulatory T cells; WT, wild type.

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hepatosteatosis and steatohepatitis as well as adipose tissue (AT) inflammation\(^1,2\) that accompany obesity. Inflammation in the liver is the determinant factor for the progression from nonalcoholic fatty liver disease (NAFLD), characterized by increased hepatic lipid accumulation, to NASH, featured by exacerbated intrahepatic inflammation, more intense steatosis, and hepatocellular injury. Increased accumulation and activation of macrophages in the liver and activation of local Kupffer cells (KCs) under obese conditions, as well as the concomitant reduction of the anti-inflammatory regulatory T cells (T regs), are important parameters in diet-induced liver steatosis and tissue damage.\(^3,4\) Additionally, the development of AT inflammation in the course of obesity further contributes to hepatosteatosis and deterioration of liver inflammation under obese conditions. More specifically, the accumulation of macrophages in AT and their interplay with infiltrated lymphocytes, such as cytotoxic CD8\(^+\) T cells, as well as the significant decrease of AT Tregs, are phenomena mediating AT inflammation.\(^1,2,5\) Consecutively, cytokines and adipokines from AT, such as leptin, interleukin (IL)-1β or IL-18, create a cross-talk with the liver and can serve as sources of extrahepatic inflammation, thereby exacerbating hepatic steatosis.\(^6,7\) Nevertheless, the information about the exact cellular and molecular mechanisms governing obesity-related inflammation in the hepatic environment, and thereby the progression of NAFLD to NASH, is scarce.

CD80 and CD86 (also designated B7.1 and B7.2) are probably the best characterized costimulatory molecules that are mainly expressed by antigen-presenting cells (APCs) and interact with CD28 and cytotoxic T lymphocyte-associated molecule 4 (CTLA-4) on T cells, mediating positive and negative signals, respectively, during T-cell activation.\(^8\) The importance of B7.1 and B7.2 signaling is defined by the fact that they participate in several immunological processes, such as T-cell proliferation, negative selection of autoreactive T cells,\(^9\) immunoglobulin class switching,\(^10\) and Treg development,\(^11\) and are therefore implicated in several pathologies, such as cancer and atherogenesis.\(^12\) The role of costimulation in regulating obesity-associated inflammation has emerged from previous studies with regard to the CD40-CD40L dyad,\(^13,14\) whereas less information on the role of B7.1 and B7.2-dependent costimulation exists.

In this study, we provide evidence that B7.1 and B7.2 have a multifaceted role in regulating obesity-related inflammatory reactions in AT and, especially, in the liver and thereby the progression from NAFLD to NASH. Performing diet-induced obesity (DIO) in B7.1/B7.2 double-deficient mice (double knockout; DKO), we observed enhanced metabolic dysregulation with glucose intolerance, IR, and deterioration of hepatosteatosis associated with increased progression to steatohepatitis, which could be ascribed to the dramatic reduction in Treg cells in B7.1/B7.2 double deficiency. Contrastingly, blocking both B7.1 and B7.2 with antibodies (Abs), but not each one alone, in wild-type (WT) mice (i.e., in the presence of normal Treg numbers) attenuated obesity-related metabolic dysregulation, improving glucose tolerance, AT inflammation, and diet-induced hepatic steatosis. Together, our findings suggest a dual role of B7 costimulation in obesity-related steatohepatitis and metabolic dysregulation governed by the presence or absence of Tregs and underline that B7.1-B7.2 costimulation may represent an interesting therapeutic target to limit obesity-related metabolic dysregulation and development of NASH.

**Materials and Methods**

**Metabolic Tests.** Blood glucose, triglycerides and cholesterol were measured in overnight fasted mice by tail-vein blood sampling and by using a glucose meter device (Accu-Chek, Roche, Mannheim, Germany) and the Accutrend Plus system (Roche, Mannheim,
Germany), respectively. For glucose tolerance test (GTT), overnight-fasted mice were injected i.p. with glucose (1g/kg). For insulin tolerance test (ITT), 5-6 h fasted mice were injected i.p. with insulin (1.5U/kg, Huminsulin, Lilly, Bad Homburg, Germany). Glucose levels were measured in both tests at 0, 15, 30, 60, 90 and 120 minutes post injection.

**Results**

**B7.1 and B7.2 Expression in Obesity.** To determine the presence and regulation of B7.1 and B7.2 in metabolic organs during obesity, we analyzed the messenger RNA (mRNA) expression of B7.1 and B7.2 in livers and gonadal AT from male WT mice fed a normal diet (ND) or a high-fat diet (HFD) for up to 18 weeks. Although there was no change in expression of B7.1 or B7.2 in total liver, we found that B7.2 was significantly up-regulated in the fraction of isolated nonparenchymal cells (NPCs), which largely represents the immune cell compartment of the liver (Supporting Fig. 1A). Moreover, both molecules were up-regulated in AT upon HFD, as well as in the stromal vascular fraction (SVF) of AT, which contains immune cells that are resident or have infiltrated in the course of DIO into AT (Supporting Fig. 1B). These findings indicate that expression of B7.1 and B7.2 is elevated in metabolic organs in the course of DIO, thereby implying a possible role of B7.1- and B7.2-dependent costimulation in the regulation of obesity-associated inflammatory processes.

**B7.1-B7.2 Double Deficiency Deteriorates Obesity-Related Metabolic Dysfunction.** Considering that the costimulatory molecules, B7.1 and B7.2, share several overlapping functional properties in regulating T-cell responses, we engaged B7 DKO mice in order to study the role of B7 costimulation in DIO. Therefore, B7.1- and B7.2-sufficient- and -deficient (DKO) male mice were fed an ND or an HFD. When fed an HFD, DKO mice displayed increased weight gain and food intake, as compared to B7.1- and B7.2-sufficient (WT) mice (Fig. 1A; Supporting Table 1). Consistently, increased weight of liver and subcutaneous (SC) AT was observed in HFD-fed DKO mice (Fig. 1B). Although SC and gonadal AT weights were also increased in DKO mice upon ND feeding, no significant difference in body-weight gain between ND-fed WT and DKO mice was observed (Fig. 1A,B). In addition, metabolic cage analysis showed increased respiratory exchange ratio (RER; Supporting Fig. 2) in DKO mice upon HFD. These data indicate decreased lipid oxidation rates in DKO mice that could account for the increased tissue weights in these mice through higher lipid deposition.

In line with these results, we found increased leptin and cholesterol levels in sera from HFD-fed DKO mice, whereas B7 double deficiency did not affect triglyceride (TG) levels (Supporting Fig. 3A). To clarify whether the hyperphagic phenotype of DKO mice was linked to higher hypothalamic inflammation, we performed gene expression analysis for immune cell markers and inflammatory cytokines in hypothalami isolated from HFD-fed WT and DKO mice (Supporting Fig. 3B). Hypothalamic expression of inflammatory markers did not differ between WT and DKO mice (Supporting Fig. 3B). Thus, the hyperphagic phenotype in DKO mice is likely a result of the increased levels of leptin with accelerated development of leptin resistance in these mice.

Consistently, obese DKO mice were more glucose intolerant, as compared to WT mice, as assessed by a glucose tolerance test (GTT; Fig. 1C). In addition, an in vivo insulin-signaling assay revealed impaired hepatic and AT insulin signaling in obese DKO mice, as indicated by decreased insulin-induced protein kinase B (AKT) phosphorylation (Supporting Fig. 4).

**B7.1-B7.2 Double Deficiency Promotes Diet-Induced Steatohepatitis.** Next, we assessed the effect of B7 double deficiency in obesity-related hepatic steatosis. Oil Red O staining, enzymatic determination of liver TG content as well as histological analysis revealed increased fat accumulation in livers from HFD-fed DKO mice, as compared to WT mice, accompanied by increased hepatocellular ballooning and histological signs of inflammation (Fig. 2A-E), which are key histological features of NASH. Consistently, the NAFLD activity score (NAS) was not only
significantly higher in obese DKO mice, as compared to WT controls (Fig. 2E), but also was >5, which correlates with the presence of NASH. That DKO mice displayed enhanced progression from hepatosteatosis to steatohepatitis was confirmed by significantly increased levels of alanine transaminase (ALT), aspartate transaminase (AST), and glutamate dehydrogenase (GLDH) in sera of HFD-fed DKO mice (Fig. 3A), thereby suggesting elevated liver cell injury in B7.1/B7.2 double deficiency. Similarly, HFD-fed DKO mice displayed higher levels of hepatic fibrotic injury, as compared to HFD-fed WT controls, as assessed by a Picric-acid Sirius red (Picrosirius) staining analysis (Fig. 3B,C). Previous studies have shown that increased hepatic profibrotic activity is linked to enhanced hepatic stellate cell (HSC) proliferation and mammalian target of rapamycin activation, including activation of the p70 S6 kinase (p70S6K). To address this point, we isolated HSCs from HFD-fed WT or DKO mice and measured HSC proliferation activity by flow cytometry (FCM) for proliferating cell nuclear antigen (PCNA) and p70S6K activity by
ribosomal protein S6 phosphorylation (Supporting Fig. 5B,C). HSCs from HFD-fed DKO mice displayed increased phosphorylation of S6 ribosomal protein, suggesting enhanced activation status and profibrotic activity of HSCs in B7 deficiency. In contrast, no difference in PCNA staining was observed.

In concordance with these data, quantitative polymerase chain reaction (qPCR) analysis showed significantly elevated expression of the lipogenic transcription factors, sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP), and a tendency for increased expression of the lipogenic enzyme, fatty acid synthase (FAS), and the fatty acid transporter, CD36 (Fig. 3D), in DKO mice in DIO. Moreover, the increased expression of glucokinase and of glucose transporter (GLUT) 2 in the
livers of DKO mice (Fig. 3D) in DIO is in keeping with the increased RER in B7.1/B7.2 double deficiency, as observed in the metabolic cage analysis (Supporting Fig. 2). Furthermore, mRNA levels of peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α and Glut4, which are important for glucose transport in stellate cells and thereby crucial for NASH-related fibrotic activity, were up-regulated in livers from obese DKO mice (Fig. 3D). These data indicate greater carbohydrate utilization, enhanced lipid deposition, and accelerated NASH development in liver of DKO mice in DIO.
Given the importance of inflammation in the progression of steatosis to NASH, we analyzed hepatic inflammation in DKO and WT mice upon DIO. In addition to increased immune cell infiltration in livers of HFD-fed DKO mice, as assessed by histological evaluation (Fig. 2E), qPCR analysis showed increased expression of IL-1β and IL-6, although the latter did not reach significance (Fig. 3D). This intrinsic reduction of Tregs in DKO mice explains the decreased numbers of hepatic Tregs in these mice, even when fed an ND. In contrast, activated KCs (defined as F4/80<sup>+</sup>CD11b<sup>+</sup> cells),<sup>19</sup> as well as dendritic cells (DCs; defined as CD11c<sup>+</sup> cells)<sup>21</sup> were increased in livers from HFD-fed DKO mice, as compared to B7-sufficient mice (Fig. 4B). Consistent with the FCM analysis data, gene expression analysis of NPCs from WT and DKO mice showed increased expression of IL-6 and a tendency to higher levels of tumor necrosis factor (TNF; Fig. 4D). These observations suggest that higher inflammation in livers of HFD-fed DKO mice, likely the result of the reduced amounts of Tregs, contributes to increased hepatic steatosis and progression to NASH under obesity-induced conditions. Together, B7 double deficiency deteriorates obesity-related hepatic steatosis and accelerates progression to steatohepatitis.

Increased AT Inflammation in DKO Mice Upon HFD. Increased AT inflammation has also been linked with obesity-related metabolic dysregulation and glucose intolerance.<sup>1,2,22</sup> Because we found deteriorated glucose tolerance in obese DKO mice (Fig. 1C), we next examined AT inflammation. FCM analysis revealed decreased Treg populations in both SC and gonadal AT of HFD-fed B7-deficient mice (Fig. 5A). Interestingly, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were reduced in both depots of white AT (Fig. 5B), implicating impaired T-cell activation resulting from the absence of B7 costimulation. Despite reduced numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in AT of DKO mice, the decrease in Tregs in these mice rather prevailed to mediate a proinflammatory environment in obese AT of these mice. In particular, increased M1 macrophages (defined as F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>−</sup>) were observed in both types of white AT (Fig. 5D), whereas total macrophages (F4/80<sup>+</sup>CD11b<sup>+</sup>) were insignificantly higher in AT of obese DKO mice (Fig. 5C). These data suggest that DKO have enhanced AT inflammation not necessarily resulting from increased macrophage accumulation, but from enhanced macrophage activation and polarization to the proinflammatory M1 phenotype, which is well in keeping with the dramatic reduction of Tregs in AT of DKO mice. Further supporting this notion, gene expression of IL-6 was significantly increased...
Fig. 5. B7 double deficiency aggravates AT inflammation. SVF cells from SC (sub) or gonadal (gon) AT of WT or DKO male mice fed an HFD for 18 weeks were isolated and analyzed by FCM. (A-D) Treg cells (defined as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) (A), CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes (B), macrophages (characterized as CD11b<sup>+</sup>F4/80<sup>+</sup>) (C), and M1 macrophages (defined as F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>-</sup>) (D) were detected. Cells were expressed as cells per gram of tissue. Treg cells were additionally expressed as percentage of CD4-positive cells (n = at least 7). Data are mean ± standard error of the mean (SEM); *P < 0.05. (E) Gonadal AT gene expression analysis from WT or DKO mice fed an ND or HFD for 18 weeks. mRNA levels of TNF, IL-6, IL-12, and monocyte chemoattractant protein 1 (MCP-1) are shown. 18S was used for normalization of mRNA expression, and the gene expression of WT ND was set as 1. n = 5/group for ND and n = 7/group for HFD. Data are mean ± SEM; *P < 0.05 for comparison between WT and DKO mice fed the same diet.
increased in DKO mice, whereas the elevation of TNF and IL-12 mRNA in gonadal AT of DKO mice almost reached significance (Fig. 5E). Taken together, B7.1/B7.2 double deficiency resulted in enhanced AT inflammation.

To further verify that DKO mice constitute an environment that promotes classical activation of macrophages (M1 polarization), we analyzed the inflammatory profile of thioglycollate-elicited peritoneal macrophages from WT and DKO mice by FCM and qPCR. Remarkably, DKO thioglycollate-elicited macrophages had an M1-skewed phenotype, as observed by increased expression of inducible nitric oxide synthase, IL-6, reduced expression of CD206, and decreased percentage of CD206+ cells (Supporting Fig. 6). Therefore, classical activation of macrophages in DKO mice likely resulting from the dramatic reduction of Tregs, seems to be an additional factor for the exacerbation of obesity-related inflammation in metabolic tissues of these mice.

Adoptive Transfer of Treg Cells Does Not Improve NASH and Metabolic Dysregulation in a B7-Lacking Environment. Our data thus far indicated that deterioration of NASH and metabolic imbalance in DKO mice was associated with a dramatic decrease of Treg cells in these mice. A previous study has suggested that adoptive transfer of Tregs is capable of reversing obesity-related metabolic dysfunction. To address whether the metabolic phenotype of DKO mice can be reversed by WT Tregs, DKO mice on DIO received weekly injections of isolated WT CD4+CD25+ Tregs or control injections from the fifth week of feeding for 6 consecutive weeks. Adoptive transfer of Tregs into DKO mice failed to reverse the metabolic phenotype of DKO mice, as assessed by GTTs (Supporting Fig. 7). Moreover, no difference in NASH development was observed in livers from DKO mice after adoptive transfer with Tregs, as compared to control (Supporting Fig. 7). Liver gene expression analysis confirmed these data by showing no difference in lipogenesis- or inflammation-related genes in livers from the two groups of mice (Supporting Fig. 7). Consistently, FCM analysis of NPCs did not show any significant differences in hepatic and splenic Treg populations between DKO mice that received Tregs or control-treated mice (data not shown). The absence of any increase in Treg populations/numbers between DKO control-treated and DKO Treg-injected mice is in line with the fact that the B7-lacking environment is highly proapoptotic for Tregs, thereby explaining that WT Tregs fail to reverse NASH and metabolic deterioration in DKO mice.

Blocking Both B7.1 and B7.2 In Vivo Improves Obesity-Related Metabolic Dysfunction. B7.1/B7.2 costimulatory molecules are important in activation of T cells, promoting inflammation in several conditions. Interestingly, our findings thus far suggested that DKO mice, despite having reduced T-cell activation (Fig. 5), rather represented a proinflammatory environment in the course of DIO, associated with reduced Treg numbers. Thereby, Dko mice displayed metabolic dysregulation in DIO, including elevated glucose intolerance and enhanced progression to hepatosteatitis. Therefore, we hypothesized that reduction of Tregs in DKO is responsible for skewing the system toward enhanced inflammation in DIO. However, adoptive transfer of WT Tregs did not reverse the development of NASH and metabolic dysregulation in DKO mice, most likely the result of increased apoptosis of Treg cells in a B7-lacking environment. In order to elucidate the role of B7 costimulation itself for obesity-related metabolic dysregulation in a normal, non-Treg-lacking environment, we performed DIO in WT mice in the presence or absence of blocking Abs to B7. Moreover, to determine whether B7.1 or B7.2 have distinct functions in DIO-related inflammation and metabolic changes, we treated HFD-fed WT mice either with anti-B7.1 or with anti-B7.2 or with the combination thereof. Mice were fed an HFD for a total of 16 weeks and injected with Abs or isotype controls (twice per week) starting at week 4 of feeding. Interestingly, treatment of WT mice in DIO with anti-B7.1 or anti-B7.2 alone did not mediate any alterations in weight gain and, importantly, in glucose tolerance, as compared to HFD-fed isotype control-treated mice (Supporting Fig. 8). However, mice injected with the combination of both anti-B7.1 and anti-B7.2 Abs displayed improved glucose tolerance and insulin sensitivity, as well as increased insulin-induced hepatic AKT phosphorylation (Supporting Fig. 9), as compared to the isotype control-treated HFD-fed WT mice, although no differences in weight gain or in the weight of metabolic tissues were observed (Fig. 6A-C). Consistently, no difference in food intake, energy expenditure or RER was observed between the two groups of mice, when metabolic cage analysis was performed (Supporting Fig. 10). As expected, exogenous administration of anti-B7.1 and anti-B7.2 Abs did not affect the numbers of Tregs in mice (Supporting Fig. 11).

We then continued to explore whether improved glucose tolerance and insulin sensitivity resulting from concomitant B7.1 and B7.2 blockade in mice were also reflected by changes in AT inflammation or liver steatosis. IL-1b and IL-6 were reduced in AT of anti-B7.1/anti-B7.2-injected mice, as revealed
Fig. 6. Blocking B7.1 and B7.2 with Abs in vivo improved obesity-related metabolic dysregulation. WT male mice were fed an HFD for a total of 16 weeks and received a combination of anti-B7.1 and anti-B7.2 Abs (anti-B7.1/B7.2 WT mice) or isotype controls (CTRL WT mice) starting at week 4 of feeding (200 μg of each Ab or isotype control were injected intraperitoneally per mouse, twice per week). (A) Body weight of HFD-fed mice treated with Abs or isotype controls is displayed in grams. (B) Glucose (GTT) and insulin tolerance tests (ITT) were performed in weeks 15 and 16 of feeding, respectively, as described under Materials and Methods. (C) SC and gonadal AT and liver weight of WT mice treated with anti-B7.1/B7.2 or isotype control Abs are displayed in grams. Data are mean ± standard error of the mean (SEM; n = at least 8); *P < 0.05. (D) Gene expression analysis of inflammatory genes and leptin was performed in gonadal AT from the two groups of mice. mRNA expression was normalized against 18S and the gene expression of isotype control-injected mice was set as 1 (n = at least 6). Data are mean ± SEM; *P < 0.05.
by qPCR analysis (Fig. 6D). These data imply that blocking of cell-cell interactions by the Ab-mediated inhibition of B7 accounts for reduced AT inflammation. Furthermore, anti-B7.1/anti-B7.2-injected mice in DIO displayed reduced hepatic steatosis and ballooning of hepatocytes as well as reduced NAS (Fig. 7A,B), whereas the fibroinflammatory injury was ameliorated in these mice, as revealed by Picrosirius staining analysis (Fig. 7C,D). The reduced lipid accumulation and liver damage phenotype of the anti-B7.1/anti-B7.2-injected mice was confirmed by qPCR analysis that displayed decreased levels of the fatty acid transporter, CD36, and also of the steatosis-related growth factor, transforming growth factor beta (TGF-β)26 (Fig. 8A). As in lymphoid organs and AT (Supporting Fig. 11 and data not shown), Treg cell population in liver was also not affected by B7.1/B7.2 blockade, as assessed by
forkhead box protein 3 (foxp3) gene expression (Fig. 8A). Interestingly, inflammatory markers, such as IL-6 and IL-1β, were significantly decreased in mice injected with anti-B7.1/anti-B7.2 (Fig. 8A).

Decreased inflammatory activation by B7.1/B7.2 blockade could be the result of inhibition of the communication of hepatocytes and/or APCs with T cells within the liver microenvironment. To verify this hypothesis, we simulated the liver environment under obese conditions in vitro and performed hepatocyte/NPC cocultures in the presence of palmitate, whereas cell-cell interactions were blocked with the combination of anti-B7.1 and anti-B7.2. Reduced IL-6, IL-1β, and TNF were detected in supernatants of cocultures when anti-B7.1 and anti-B7.2 were applied, as compared to the isotype control treated cocultures (Fig. 8B). These data imply that B7 costimulation could mediate intercellular communication between hepatocytes and immune cells, thereby potentially triggering hepatic inflammation in DIO.

Taken together, concomitant blockade of B7.1 and B7.2 in the presence of Tregs improves glucose tolerance, insulin sensitivity, hepatic steatosis, as well as AT and liver inflammation, thus suggesting B7 costimulation itself contributes to obesity-related inflammation and metabolic dysregulation.

Discussion

Costimulatory molecules play a major role in the intercellular communication of adaptive immune cells with APCs, innate immune cells, or parenchymal cells.8,27,28 A plethora of cell-cell interactions take place in liver and AT in the course of obesity, regulating inflammation and thereby the development of steatohepatitis and IR.1,2,20,29,30 Here, we identified B7-dependent costimulation as a major regulator of
obesity-related inflammatory processes and metabolic dysregulation.\textsuperscript{1,2} B7.1/B7.2 double deficiency exacerbated the development of NASH and metabolic dysregulation upon DIO, a phenotype attributed to the absence of Treg cells in liver and AT of these mice. In contrast, Ab-mediated blockade of B7 interactions under conditions, where regulatory T cells are present, protected mice from the development of obesity-related pathologies and, especially, NASH.

In the course of obesity, DKO mice displayed a deteriorated metabolic phenotype. Importantly, DKO mice displayed increased hepatic steatosis, hepatocellular ballooning, deteriorated hepatic insulin signaling, and enhanced progression to NASH. Inflammation constitutes the second hit in the prevailing two-hit theory for the pathogenesis of NASH, in which lipid accumulation is followed by a second stimulus that triggers the evolution of steatosis to steatitis.\textsuperscript{4,19,20} Activated KCs in the liver mediate obesity-related liver inflammation, mainly by producing cytokines such as TNF, IL-6, and IL-1\textsubscript{b}.\textsuperscript{20,29,31} Likewise, the reduction in inflammation, mainly by producing cytokines such as TNF, IL-6, and IL-1\textsubscript{b}.\textsuperscript{20,29,31} The ploproliferative hepatic features associated with NASH development were exacerbated in B7 double deficiency. The dramatic decrease in Treg numbers in blood and lymphoid tissues of DKO mice resulted from the absolute requirement of B7 costimulation for Treg survival, and proliferation\textsuperscript{11,35} resulted in the absence of Tregs from metabolic organs (liver and AT). The B7-deficient environment is “toxic” to Tregs, as was evidenced by the failure of adoptive transfer of WT Tregs to reverse the metabolic phenotype of DKO mice, as compared to WT mice in DIO.\textsuperscript{25} Consistent with increased Treg apoptosis in the B7-negative environment,\textsuperscript{24,25} no increase in Treg numbers was found in the lymphoid and metabolic organs of B7-deficient mice upon adoptive Treg transfer. Together, because of the absence of Tregs, deficiency of B7 costimulation promoted a proinflammatory environment in the liver with increased numbers of activated KCs and enhanced proinflammatory gene expression. Our findings are in line with those of Ma et al. that demonstrated a connection between Treg reduction and the development of liver steatosis under obese conditions.\textsuperscript{4} Together, the early and exacerbated NASH development in B7.1/B7.2 double deficiency also indicates that DKO may represent a feasible mouse model for studying NASH in future studies.

Consistently, HFD-fed DKO mice displayed higher levels of hepatic fibroinflammatory injury. Isolated HSC from DKO mice displayed increased phosphorylation of S6 ribosomal protein, suggesting enhanced profibrotic activity,\textsuperscript{16-18} whereas we did not find significantly elevated HSC proliferation as a result of B7 deficiency. The latter could be because of the fact that only one time point (15 weeks on HFD) was used for proliferation analysis of isolated HSCs. A detailed time-kinetic analysis comparing stellate cell proliferation and activation in WT and DKO mice on an HFD and ND for different time points would be necessary to fully address this issue in a future study.

Accumulating evidence underlines the role of leptin in NASH development mainly through immune cell activation in NAFLD.\textsuperscript{34,35} The increased levels of leptin accompanied by reduced Treg numbers in B7 DKO mice are in line with the already established reciprocal relationship between leptin levels and Treg proliferation.\textsuperscript{36-38} Thus, in our study, the enhanced leptin levels in B7 DKO mice likely contributed to the progression to steatohepatitis in these mice.

In addition to steatohepatitis, metabolic parameters, such as glucose tolerance or insulin signaling, were worse in B7-deficient mice. Metabolic deterioration in B7-deficient mice was associated with significantly higher AT inflammation, as assessed by the enhanced M1 polarization of macrophages in AT and increased expression of proinflammatory cytokines, such as TNF and IL-6, that act to mediate AT IR.\textsuperscript{2,31} Interestingly, the AT environment in DKO mice was proinflammatory despite reduced accumulation of effector T cells (CD4\textsuperscript{+} and CD8\textsuperscript{+} cells), as B7 costimulation is an important effector for T-cell activation. Together, our data suggest that, although costimulation is indispensable for T-cell activation, the diminution of Tregs in metabolic organs of DKO is hierarchically the driving force triggering liver and AT inflammation in DIO, thereby leading to steatohepatitis and glucose intolerance.

To further support the notion that the dramatic reduction in Tregs accounted for aggravation of steatohepatitis and metabolic deterioration in DKO mice and could override any beneficial effects of disruption of B7-dependent costimulation, we blocked B7 interactions under conditions where Tregs were present. To this end, we exogenously administered anti-B7.1 and anti-B7.2 Abs into WT mice in DIO. The use of B7 blockade was the only possible experimental approach to address Treg-independent functions of B7 costimulation in DIO, given our finding that adoptive transfer of WT Treg cells was not operative in the B7-negative environment, although this approach has been previously
engaged to ameliorate obesity-related AT inflammation and metabolic dysregulation in B7-sufficient WT settings.\textsuperscript{23,39} The improvement of metabolic dysregulation and reduced hepatosteatosis/steatohepatitis by concomitant Ab-mediated inhibition of both B7.1 and B7.2 in DIO, but not by inhibiting each one alone, suggests a synergistic action of B7.1 and B7.2 to promote obesity-related inflammation. B7 costimulation was found to mediate interactions between hepatocytes and NPCs, which are important for the initiation and progress of inflammation in the intrahepatic environment.\textsuperscript{40} In particular, anti-B7.1/B7.2 blockade reduced the expression of inflammatory cytokines in hepatocyte/NPC cocultures. Moreover, blocking B7 interactions alleviated AT inflammation, in keeping with recent studies that identified adipocytes as APCs.\textsuperscript{41} A previous study has manipulated the B7/CD28/CTLA-4 costimulation by using CTLA-4/immunoglobulin; this treatment did not significantly improve steatohepatitis or metabolic dysregulation in DIO.\textsuperscript{42} While our article was in preparation, a study appeared showing that B7 double deficiency is linked to enhanced AT inflammation and IR; in contrast, this study did not report much on the hepatic phenotype of B7 double deficiency.\textsuperscript{43} Based on their findings with DKO mice, these investigators concluded that B7 costimulation protects from IR. However, our approach to block B7.1 and B7.2 in the presence of Tregs in WT mice uncovered that B7 costimulation, in fact, promotes obesity-related metabolic dysregulation and NASH. Therefore, we conclude that intercellular B7 costimulatory interactions contribute to the exacerbation of inflammation in liver and AT during DIO. However, in DKO mice, the absence of Tregs simply masked any beneficial anti-inflammatory effects that could derive from inactivation of B7 costimulation.

In conclusion, the present study identified that B7.1 and B7.2 costimulation plays important roles in obesity-related inflammation, glucose intolerance, and hepatosteatitis. Genetic inactivation and Ab inhibition of CD80/CD86 costimulatory molecules affect obesity-related steatohepatitis and metabolic dysregulation in an opposite fashion, which is orchestrated by the numbers and function of Tregs. Our findings not only underline the importance of Tregs as a master regulator of obesity-driven liver inflammation, but also indicate that inhibition of B7 costimulation may provide a novel platform for potential therapeutic manipulation for the prevention of NASH and likely other obesity-related complications.

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Supporting Information

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