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Obstructive Jaundice Expands Intrahepatic Regulatory T Cells, Which Impair Liver T Lymphocyte Function but Modulate Liver Cholestasis and Fibrosis

Steven C. Katz,*‡,+ Kristin Ryan, † Naseem Ahmed, † George Plitas,* Umer I. Chaudhry,* T. Peter Kingham,* Seema Naheed, † Cang Nguyen, † Ponnandai Somasundar, †‡ N. Joseph Espat,*‡ Richard P. Junghans,†‡ and Ronald P. DeMatteo*

Although obstructive jaundice has been associated with a predisposition toward infections, the effects of bile duct ligation (BDL) on bulk intrahepatic T cells have not been clearly defined. The aim of this study was to determine the consequences of BDL on liver T cell phenotype and function. After BDL in mice, we found that bulk liver T cells were less responsive to allogeneic or syngeneic Ag-loaded dendritic cells. Spleen T cell function was not affected, and the viability of liver T cells was preserved. BDL expanded the number of CD4+CD25+Foxp3+ regulatory T cells (Treg), which were anergic to direct CD3 stimulation and mediated T cell suppression in vitro. Adoptively transferred CD4+CD25+ T cells were converted into Treg within the liver after BDL. In vivo depletion of Treg after BDL restored bulk liver T cell function but exacerbated the degrees of inflammatory cytokine production, cholestasis, and hepatic fibrosis. Thus, BDL expands liver Treg, which reduce the function of bulk intrahepatic T cells yet limit liver injury. *The Journal of Immunology, 2011, 187: 1150–1156.

Bile duct ligation (BDL) is a well-established model of obstructive jaundice (1), a condition known to alter immunity and physiology. Obstructive jaundice leads to intrahepatic inflammation and fibrosis. Jaundiced patients are at increased risk for complications after surgical procedures (2–6) and suffer from significant metabolic (7, 8) and immunologic derangements (9–11), including altered proliferative responses among splenic lymphocytes and PBLs (10, 12). The effects of BDL on bulk liver T cells and regulatory T cells (Treg) have not been defined. Treg have recently been suggested to contribute to alterations in Treg immunobiology. In this study, we investigated the effects of BDL on murine liver T cells. The results demonstrate that BDL alters the function of bulk liver T cells, accompanied by an expansion of liver Treg. Conversion of CD4+CD25+ T cells to Treg in the liver was demonstrated, and depletion of Treg led to recovery of bulk liver T cell alloresponsiveness. Treg depletion also led to increased levels of cholestasis and intrahepatic inflammation. Therefore, liver Treg may play a dual role in the setting of obstructive jaundice by suppressing T cell function while limiting cholestasis and hepatic fibrosis.

**Materials and Methods**

**Mice**

Adult 6- to 10-wk-old male C57BL/6 (B6, H-2Kb) and BALB/c (H-2Kd) mice were purchased from Taconic Farms (Germantown, NY). OT-II TCR transgenic Rag2−/− mice on a B6 background were also obtained from Taconic. Foxp3-GFP (C.Cg-Foxp3tm2Tch/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in a pathogen-free facility at the Sloan-Kettering Institute or at Roger Williams Hospital. Procedures were approved by the institutional animal care and use committees. Histologic sections with routine staining were performed at the Boston University Medical Center Experimental Pathology Service Core.

**Surgical procedures**

Mice were anesthetized with i.p. administration of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Lloyd Laboratories, Shenandoah, IA) or with inhaled anesthetic. The abdomens were then shaved and prepared in sterile fashion. An upper-midline laparotomy incision was made, and the common bile duct was ligated with 6-0 silk suture (Ethicon, Somerville, NJ). The peritoneum and fascia were closed with absorbable suture material, followed by skin clips. All steps excluding ligation of the bile duct were performed for sham operations. Criteria for successful BDL at the time of animal sacrifice included jaundiced soft tissues, patchy liver discoloration, and biliary tree dilation. More than 95% of operations met our criteria for successful BDL.

**Cell preparation**

Liver nonparenchymal cells (NPC) were isolated as previously described, with modifications (17). Briefly, animals were euthanized, and the portal...
vein was injected with 3 ml 1% (w/v) collagenase IV (Sigma, St. Louis, MO) in HBSS. For serum chemistry analysis, blood was harvested from the heart prior to portal vein infusion. The liver was mechanically disrupted prior to incubation in 10 ml 1% collagenase at 37°C for 20 min. The resulting cell suspension was passed through sterile 100-μm nylon mesh filters (Falcon; BD Biosciences) and centrifuged three times at 30 g × 5 min to remove hepatocytes. The specimens were pelleted (300 × g for 7 min), red cells were lysed, and the remaining cells were washed in complete media (RPMI 1640, 10% FBS, 2 mM L-glutamine, 0.1% 2-mercaptoethanol, 100 μg/ml penicillin, 100 μg/ml streptomycin). The pellet containing NPC was resuspended in 3.0 ml RPMI 1640 and then combined with 2.0 ml 40% (w/v) Optiprep (Sigma) to remove debris and enrich the cells. The suspension was layered under 4 ml GBSS and spun at 97%.

Flow cytometry

Flow cytometry was performed on FACSscan or LSR-II flow cytometers (BD Biosciences). Voltages were set based on unstained cells, and compensation was adjusted using single-stained controls. Samples were incubated with Fc block prior to staining with Ab against CD3 (145-2C11), CD4 (RM4-4), CD8 (53-6.7), CD25 (2A3), CD69 (FN50), NK1.1 (PK136), and CD1d/α-galactosylceramide (αGalCer; Miltenyi Biotec). Cells were then fractionated based on Thy1.2 (CD90.2) or CD11c expression using immunomagnetic beads (Miltenyi Biotec, Auburn, CA) and positive selection columns (Miltenyi Biotec). Dead cells were excluded with 7-aminoactinomycin D (BD Biosciences), anti-GITR (DTA1; BD), or normal saline i.p. on days −1, 0, 5, and 7 relative to BD or sham. For Treg conversion assays, CD4+CD25+ B6 splenocytes were isolated using immunomagnetic beads (Miltenyi Biotec), and 1 × 10^6 CFSE+CD4+CD25+Foxp3+ for phenotype). The purity of sorted cell populations was typically >97%.

**T cell stimulation assays**

MLRs were performed as previously described (16) by culturing splenic DC from BALB/c mice with B6 T cells. CD4+CD25− T cells were excluded by FACS or added in varying concentrations for some experiments. When CFSE was used, T cell stimulation in the absence of APCs, 1 × 10^3 bulk T cells or 1 × 10^6 CD4+CD25− or CD4+CD25+ T cells were cultured in 96-well flat-bottom plates (Falcon) with anti-CD28 (20 μg/ml) and plate-bound anti-CD3 (BD Biosciences). Cell proliferation was measured by pulsing with [H]thymidine (1 μCi/well) on day 3 or flow cytometry to measure CFSE dissolution. When CFSE was used, cells were labeled according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Supernatant was harvested from triplicate wells for cytokine measurement with cytokine bead array (BD Biosciences).

**In vivo experiments**

Treg were targeted by administration of 100 μg anti-CD25 (PC61; BD Biosciences), anti-GITR (DTA1; BD), or normal saline i.p. on days −1, 0, 5, and 7 relative to BD or sham. For Treg conversion assays, CD4+CD25+ B6 splenocytes were isolated using immunomagnetic beads (Miltenyi Biotec), and 1 × 10^6 CFSE+CD4+CD25+ splenocytes were then adoptively transferred via portal vein after BD or sham. Portal vein injections were carried out via 30-gauge needles using volumes of 200 μl. A 3 × 3 mm piece of Surgicel (Ethicon) was applied to the portal vein puncture site and direct pressure applied for 60 s to achieve hemostasis.

**Statistics**

Statistical analyses were performed using a two-tailed t test (P<0.05).
a suppressive effect. We focused on conventional liver CD4 T cells for this experiment given our previous finding that these cells are suppressed within their native environment (16). Conventional CD4 T cells were prepared by excluding potentially suppressive NKT, Treg, and γδ T cells. Treg were excluded by elimination of CD4+CD25+ cells. We found that the alloproliferation of conventional CD4 cells to direct CD3 stimulation was not diminished after BDL (Fig. 1D). Similarly, the responsiveness of liver conventional CD4 cells to direct CD3 stimulation was not significantly affected by BDL (Fig. 1D). Of note, annexin V staining and DAPI uptake by bulk liver T cells were not altered after BDL 7 d after operation (data not shown).

**BDL results in an expansion of liver Treg**

Having found that BDL impaired the bulk liver T cell population but not conventional liver CD4 T cells, we speculated that a subpopulation of liver T cells was mediating a suppressive effect. We focused on liver Treg given their known suppressive properties in other models (14, 22). Whereas the overall number of liver NPC and bulk T cells was not affected by BDL, the proportion of CD4+CD25+ liver T cells was also observed in the livers of OT-II mice after BDL (data not shown). Although we found that the frequency of CD4+CD25+ liver T cells was consistently increased after BDL, we considered that CD25 expression may be upregulated nonspecifically. We measured the percentage of CD4+CD25+ liver T cells that also expressed Foxp3 and GITR, which are indicators of Treg in mice (19, 23). The majority of CD4+CD25+ liver T cells expressed Foxp3 and GITR (Fig. 2B), and we also confirmed liver Treg expansion in Foxp3-GFP transgenic mice (Fig. 2C). The Treg expansion in the liver after BDL was verified by a significant increase in the absolute cell count as well (Fig. 2D). We also determined that the majority of liver CD4+CD25+ T cells were Treg on the basis of Foxp3 expression in OT-II mice (data not shown), consistent with the work of other groups (24, 25).

**CD4+CD25+ T cells convert to Treg within the liver after BDL**

The above data demonstrate liver Treg are expanded after BDL. To determine if Treg are derived from peripheral conversion within the liver, CFSE-labeled CD4+CD25+ syngeneic T cells were adoptively transferred via the portal vein during BDL or sham procedures. On day 7, livers were harvested, and adoptively transferred

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**FIGURE 2.** BDL expands liver Treg in B6 mice. A, Flow cytometry was used to determine the frequency of CD25+ lymphocytes among the liver CD4 cell population (CD3+CD4+NK-γδT) at 7 d after BDL. B, To determine the proportion of the CD4+CD25+ liver T cell population that was actually Treg, we measured the levels of Foxp3 and GITR expression among these cells in all three groups. C, Liver Treg expansion assessed by flow cytometry among B6 mice, labeled with CFSE, and then (1×10⁶) adoptively transferred via the portal vein at the time of BDL or sham. After 7 d, liver T cells were harvested and analyzed by flow cytometry. A, The proportion of CFSE+ adoptively transferred cells among bulk (Thy1.2+) liver T cells was significantly higher after BDL. B, To determine the proportion of adoptively transferred cells that adopted a Treg phenotype, we measured CD25 expression among CFSE+ cells. D, Foxp3 expression confirmed in isolated, converted CD4+CD25+ T cells. Representative of two repetitions with three mice per group. *p < 0.05.
CFSE+ cells were analyzed by flow cytometry. Jaundiced mice had a significantly higher percentage of CFSE+ adoptively transferred cells among bulk liver T cells (Fig. 3A). Compared with sham livers, a nearly 2-fold higher proportion of adoptively transferred CFSE+CD4+CD25+ T cells converted to a Treg phenotype (CD4+CD25+) after BDL (Fig. 3B, 3C). We confirmed that >70% of converted CD4+CD25+ cells expressed Foxp3 in both BDL and sham groups (Fig. 3D).

**Liver CD4+CD25+ T cells demonstrate suppressive function after BDL**

We next sought to determine if CD4+CD25+ liver T cells after BDL possessed Treg functional properties. We chose to measure Treg inhibition of MLR, a known suppressive effect of Treg (26). To this end, we cultured CD4+CD25− T cells with varying numbers of CD4+CD25+ T cells from BDL livers in the presence of allogeneic DC. We found that CD4+CD25− T cells, isolated from BDL-treated animals, were able to suppress the response of CD4+ CD25− T cells to allogeneic DC (Fig. 4A). CD4+CD25+ liver T cells isolated from control animals did not demonstrate suppressive function (data not shown). To validate further these findings, we performed the converse experiment by eliminating CD4+CD25+ T cells from bulk liver T cells purified from jaundiced animals. Removal of CD4+CD25+ T cells from the bulk T cell population led to a restoration of alloproliferation (Fig. 4B). Liver CD4+CD25+ T cells from BDL mice also demonstrated relative hyporesponsiveness to direct CD3/CD28 stimulation compared with CD4+CD25− T cells from the same animals (data not shown).

**In vivo depletion of Treg results in recovery of bulk liver T cell function**

To determine if the presence of Treg in the liver after BDL was necessary for ex vivo bulk liver T cell dysfunction, we depleted Treg in mice subjected to BDL. Anti-CD25 or anti-GITR Ab was administered i.p. on days −1, 0, 5, and 7 relative to BDL or sham operations. On day 8, animals were sacrificed, and bulk liver T cells were isolated and labeled with CFSE prior to stimulation with allogeneic DC. The efficiency of depleting Treg with either anti-CD25 or anti-GITR was confirmed (Fig. 5A). Bulk liver T cells isolated from animals treated with anti-CD25 demonstrated improved responsiveness to stimulation by allogeneic DC compared with control mice with intact Treg populations (Fig. 5B). We confirmed these findings by measuring IFN-γ levels in the supernatant from the MLR assays. Bulk liver T cells produced significantly higher levels of IFN-γ when isolated from jaundiced mice having been treated with anti-CD25 (Fig. 5C). Treatment of mice with anti-GITR also resulted in markedly enhanced ex vivo bulk liver T cell alloresponsiveness after BDL (Fig. 5B). The positive functional effects of anti-CD25 and anti-GITR treatments were not apparent in bulk liver T cells isolated from sham mice (data not shown).
Depletion of Treg promotes a proinflammatory cytokine profile among liver T cells

To determine the physiologic impact of Treg expansion in the setting of obstructive jaundice, we measured cytokine production by liver T cells with or without Treg depletion. When Treg were depleted from B6 mice subjected to BDL, bulk liver T cells made significantly lower levels of IL-10 after allogeneic stimulation (Fig. 6A). In contrast, bulk liver T cell IL-6 production was enhanced after Treg depletion in jaundiced animals (Fig. 6B).

Treg depletion exacerbates cholestasis and hepatic fibrosis after BDL

We next determined if the more inflammatory cytokine profile of liver T cells after Treg depletion in jaundiced animals was associated with measurable increases in intrahepatic cholestasis and fibrosis. After BDL, serum bilirubin levels were significantly higher when Treg were depleted (Fig. 7A). Alkaline phosphatase levels increased nearly 2-fold after BDL as a consequence of Treg depletion (Fig. 7B). The exacerbation of intrahepatic inflammation associated with Treg depletion was confirmed histologically through demonstration of greater degrees of infiltration by inflammatory cells and periportal fibrosis using Mallory’s trichrome stain (Fig. 7C).

Discussion

Our data demonstrate that obstructive jaundice suppresses bulk liver T cell function in association with an expansion of Treg, which modulate the extent of cholestasis and fibrosis. The expansion of liver Treg was due, at least in part, to conversion of CD4+CD25+ T cells into cells with a Treg phenotype (CD4+CD25+Foxp3+). The suppressive function of expanded liver Treg was demonstrated in vitro and in vivo. Although Treg expansion adversely affected the function of bulk liver T cells, Treg protected the liver parenchyma by limiting the degrees of cholestasis and fibrosis, in addition to altering cytokine production by bulk liver T cells. Taken together, our results implicate liver Treg as mediators of immunosuppression and modulators of liver injury in the setting of biliary obstruction.

Biliary obstruction has been associated with numerous systemic and intrahepatic derangements, including immunologic dysfunction (2–4, 7, 8, 27, 28). Whereas jaundice has been shown to alter the function of splenic and PBLs, the effect of biliary obstruction on bulk intrahepatic T cells is less clear. Our data demonstrate that the function of bulk liver T cells is suppressed after BDL in association with an expansion of intrahepatic Treg. As depletion of Treg with either anti-CD25 or anti-GITR led to a restoration of bulk liver T cell function when tested ex vivo, we speculate that Treg play a critical role in mediating suppression of liver T cells in the setting of obstructive jaundice. Although depletion of Treg with either anti-CD25 (29) or anti-GITR (30) restored bulk liver T cell function, the degree of intrahepatic inflammation was exacerbated. These functional results corroborate our phenotype studies suggesting that the majority of expanded CD4+CD25+ liver
T cells are in fact Treg (19, 23, 31). We did not find a significant difference between anti-CD25 and anti-GITR in terms of Treg depletion efficiency or restoration of T cell allosresponsiveness (Fig. 5).

The suppressive function of the expanded liver CD4<sup>+</sup>CD25<sup>+</sup> T cells was confirmed by multiple, independent in vivo and in vitro experiments. Liver CD4<sup>+</sup>CD25<sup>+</sup> T cells from BDL livers demonstrated the ability to suppress the in vitro proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells in response to DC (Fig. 4A, 4B) and anti-CD3/anti-CD28 (data not shown), similar to prior reports (32–34). CD4<sup>+</sup>CD25<sup>+</sup> liver T cells isolated from control animals did not demonstrate suppression (data not shown). Enhanced Treg suppressor function in the setting of intrahepatic inflammation was demonstrated in a study involving Con A-induced liver injury (14). Therefore, in the setting of either BDL or Con A-induced liver injury, liver Treg may acquire enhanced suppressive function. This is also supported by our in vivo experiment in which Treg depletion led to enhanced ex vivo bulk liver T cell function in BDL but not sham mice.

Suppression of bulk liver T cell function after BDL may be disadvantageous from the standpoint of vulnerability to infection, but the expansion of Treg may benefit the host by ameliorating the degree of intrahepatic cholestasis and fibrosis. Depletion of Treg in mice subjected to BDL resulted in significantly higher levels of serum alkaline phosphatase and bilirubin, suggesting that Treg modulated the extent of intrahepatic inflammation (Fig. 7). Histologic analysis of liver tissue after BDL with or without Treg depletion revealed that the degree of fibrosis was higher in mice treated with the anti-CD25 Ab. Similarly, treatment with anti-GITR resulted in increased levels of intrahepatic fibrosis (data not shown). The increased damage to liver tissue after Treg depletion in jaundiced mice was accompanied by increased IL-6 and decreased IL-10 production by bulk liver T cells (Fig. 6). Although other cytokines may ultimately be implicated as well, our data suggest that alterations in IL-6 and IL-10 levels contribute to the modulation of intrahepatic inflammation by Treg in the setting of biliary obstruction.

Although we cannot fully account for the source of expanded Treg in jaundiced mice, our data indicate that intrahepatic conversion of CD4<sup>+</sup>CD25<sup>-</sup> T cells into Treg occurred. A significantly higher proportion of adoptively transferred CD4<sup>+</sup>CD25<sup>-</sup> T cells converted to Treg after BDL compared with that in sham based on expression of CD25 and Foxp3 (Fig. 6). Our data do not, however, exclude that migration of Treg into the liver occurred as well. Treg have been demonstrated to migrate across the hepatic sinusoidal endothelium, and therefore an influx of Treg from extrahepatic sources may have contributed to the expansion found in our model of obstructive jaundice (35).

Our findings with BDL and depletion of Treg suggest that liver Treg mediated suppressive effects in vivo within the liver. Although these data lend support to the importance of Treg in mediating liver T cell suppression in the setting of obstructive jaundice, other cell types likely contribute to liver T cell suppression as well. Recently, invariant NKT cells have been purported to modulate the inflammatory milieu after BDL (36). We did not find an increase in the proportion of NKT, but BDL resulted in an expansion of γδ T cells (data not shown), which we previously demonstrated to have suppressive properties (16). We also did not directly study the effect of BDL on other liver NPC such as DC, LSEC, and Kupffer cells. It is possible that these cell types may mediate additional effects, as we have found DC to become activated after BDL (1). There are other potential explanations for our findings that liver T cell function is altered after BDL. Bile acids and bilirubin accumulate in the liver after BDL and mediate direct effects (37, 38). It is likely that the functional changes observed among liver T cells are due to several alterations within the intrahepatic environment, and an expansion of Treg represents but one contributing factor. In addition, Treg may also have direct or indirect suppressive effects on DC through alterations in the intrahepatic cytokine milieu (39).

BDL has been associated with an increase in systemic inflammatory cytokines (40) and bacterial translocation (28). We did find bacteria to be present in the blood and bile after BDL, in addition to enhanced production of IL-6 and MCP-1 by bulk liver T cells (data not shown). Whether Treg depletion with the associated alterations in IL-6 and IL-10 levels prevents bacteremia and bactiuria remains to be determined. Although biliary obstruction appears to have systemic effects, alterations in T cell function or phenotype were specific to the liver in our study. Previous reports have indicated that BDL results in functional impairment of both spleen and peripheral blood T lymphocytes (10, 12). However, we did not detect functional changes in spleen T cells after BDL (Fig. 1B). Other groups studied rats instead of mice (12) or studied the response of spleen T cells to phytohemagglutinin and not DC (10–12). In addition, our method of T cell isolation from the liver or spleen differed from that of other groups. Consistent with our data, another group reported that inflammation resulted in Treg expansion in the liver but not the spleen (41). Given that the inflammatory consequences of BDL (Fig. 6) are predominantly detected in the liver, it is not surprising that the immunologic sequelae are most pronounced in the liver as well.

Taken together, our data demonstrate that BDL in mice affects the function of intrahepatic T cells in association with an expansion of Treg. In particular, bulk liver T cells have a diminished response to DC. These alterations are accompanied by an increase in Treg, which was found to be related to a conversion of CD4<sup>+</sup>CD25<sup>-</sup> cells. Depletion of Treg led to recovery of bulk liver T cell function but an increase in cholestasis and fibrosis, which were associated with significant changes in IL-6 and IL-10 production. These findings suggest that, in the setting of obstructive jaundice, liver Treg may negatively impact intrahepatic immunity while limiting the detrimental effects of the associated inflammatory response. A more detailed understanding of the mechanisms underlying these observations may allow for clinically useful immunomodulatory strategies in the setting of biliary occlusion.

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Disclosures
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