Biliary Obstruction Selectively Expands and Activates Liver Myeloid Dendritic Cells

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Biliary Obstruction Selectively Expands and Activates Liver Myeloid Dendritic Cells


Obstructive jaundice is associated with immunologic derangements and hepatic inflammation and fibrosis. Because dendritic cells (DCs) play a major role in immune regulation, we hypothesized that the immunosuppression associated with jaundice may result from the functional impairment of liver DCs. We found that bile duct ligation (BDL) in mice expanded the myeloid subtype of liver DCs from 20 to 80% of total DCs and increased their absolute number by >15-fold. Liver myeloid DCs following BDL, but not sham laparotomy, had increased Ag uptake in vivo, high IL-6 secretion in response to LPS, and enhanced ability to activate T cells. The effects of BDL were specific to liver DCs, as spleen DCs were not affected. Expansion of liver myeloid DCs depended on Gr-1+ cells, and we implicated monocyte chemotactic protein-1 as a potential mediator. Thus, obstructive jaundice selectively expands liver myeloid DCs that are highly functional and unlikely to be involved with impaired host immune responses. The Journal of Immunology, 2006, 176: 7189–7195.

O bstructive jaundice has many etiologies, the most common of which are extrahepatic biliary lithiasis and neoplasm. The consequences of jaundice are complex and involve a variety of physiologic derangements, including immunosuppression as well as hepatic fibrosis. In particular, patients with jaundice have a predisposition to infection and increased morbidity and mortality (1, 2). Altered function of a number of immune effector cells has been implicated in the pathophysiology of jaundice. Experimentally, there is reduced mitogen-induced lymphocyte proliferation (3), and it has been demonstrated that bilirubin inhibits the induction of CTL activity, possibly via an impaired response to IL-2 (4). The reticuloendothelial system in patients with jaundice takes up less microaggregated albumin administered i.v., which may explain the deficiency in endotoxin clearance (5). Reduced bacterial clearance by Kupffer cells is believed to result from their high production of IL-10 (6). However, in contrast to its effects on T cells and Kupffer cells, jaundice increases the function of neutrophils (7, 8).

The liver plays a major role in peripheral tolerance and immunity under normal conditions (9, 10). At the cellular level, dendritic cells (DCs) are thought to be the critical mediators of immunologic outcomes, given that they can capture and process Ag and activate or tolerize effector cells (11, 12). To begin to ascertain the importance liver DCs in immunity, we (13) and others (14–17) have previously characterized the subsets of liver DCs in normal mice. We identified liver plasmacytoid DCs expressing B220 as well as four other distinct subtypes of liver DCs based on CD11b and CD8α staining. We found that classical myeloid (CD11c+CD11b+CD8α+) and lymphoid (CD11c+CD11b−CD8α+) DCs each comprise ~20% of total liver DCs. Whereas bulk liver DCs were substantially less functional than bulk splenic DCs, liver lymphoid and myeloid DCs had equal function compared with their splenic counterparts. However, there are few data on the function of liver DCs in pathologic states. Because of the critical role of DCs in immune regulation, we hypothesized that immunosuppression associated with obstructive jaundice is due to an impairment of hepatic DC function. We tested this postulate in a murine model of bile duct ligation (BDL).

Materials and Methods

Animals and procedures

Male 4- to 8-wk-old C57BL/6 (B6, H-2Kb), BALB/c (H-2Kd), and Rag2−/− OT-II (OT-II) OVA TCR-transgenic mice (H-2Kb) were purchased from Taconic Farms. IL-6−/− and TNF−/− mice on a B6 background were each purchased from The Jackson Laboratory. BDL was performed under ketamine/xylazine anesthesia via a midline laparotomy. With a surgical dissecting microscope, the common bile duct was doubly ligated using 7-0 Prolene suture. Sham-operated animals went through an identical laparotomy and liver manipulation without BDL. To deplete Gr-1+ cells in vivo, mice were administered four daily i.p. injections (0.2 mg) of either a Gr-1 depleting Ab (anti-Gr-1; Rb6–8C5) or an irrelevant (anti-mouse IgM; 331.2.3) Ab (both from the mAb Core Facility, Sloan-Kettering Institute, New York, NY). Mice that were obviously ill or moribund after sham laparotomy or BDL were excluded. The Institutional Animal Care and Use Committee of the Sloan-Kettering Institute approved all procedures.

DC isolation and culture

Liver nonparenchymal cells (NPCs) were isolated as detailed (13) with modifications. Briefly, after animals were euthanized by CO2 inhalation, the portal vein was cannulated and injected with 2 ml of 1% (w/v) collagenase IV (Sigma-Aldrich) in PBS. After mechanical disruption, the cell suspension was passed through a 100-μm filter and then centrifuged three times at 30 x g to remove hepatocytes. The remaining NPCs were pelleted, and, after RBC lysis, CD45+ cells were purified using immunomagnetic beads according to the manufacturer’s protocol (Miltenyi Biotec). CD45+ NPCs were then used for either functional assays or stained for cytometric analysis or FACS sorting. Cells for culture were plated at various concentrations in RPMI 1640 with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM 2-ME, and, in some cases, IL-6 (10 ng/ml) or TNF-α (20 ng/ml) (R&D Systems). The cells were then stained with fluorescence conjugated Abs to CD68α, NK1.1, CD11b, Gr-1, and CD11c for further separation of subtypes using a MoFlo

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3 Abbreviations used in this paper: DC, dendritic cell; BDL, bile duct ligation; NPC, nonparenchymal cell.
cell sorter (DakoCytomation). Myeloid DCs were isolated based on their expression of CD11c and CD11b, whereas NK1.1<sup>+</sup> and CD8α<sup>+</sup> cells were excluded. Care was taken to exclude highly autofluorescent cells during FACS sorting, and sorted cells were typically >97% pure for the desired set of surface markers. To obtain sufficient cells for FACS analysis and for functional studies after sorting, cells typically from 3 to 6 mice per group were pooled.

**Flow cytometry**

Five- and six-color flow cytometry was performed on either a FACSCalibur, modified FACSscan (BD Biosciences) or a CYAN (DakoCytomation) flow cytometer. Voltages were based on unstained cells, and compensation was set using single-stained positive controls for each color. We stained 1 x 10<sup>6</sup> cells per tube with 1 μg of FITC, PE, PerCP, allophycocyanin, or allophycocyanin-Cy7-conjugated Ab (BD Pharmingen, except as indicated) after blocking with 1 μg of anti-FcγRIII/II Ab (clone 2.4G2; mAb Core Facility, Sloan-Kettering Institute). In some cases dead cells, which typically comprised 3-7% of freshly isolated CD4<sup>+</sup> hepatic NPCs, were excluded with either 7-aminoactinomycin D (BD Biosciences) or 4,6-diamidino-2-phenylindole, dilactate (Invitrogen Life Sciences). Cells were stained for the DC marker CD11c (HL3), the lineage markers CD11b (M1/70), Gr-1 (RB6-8C5), CD8a (53-6.7), NK1.1 (PK136), and MHC class II (I-Ab), and the costimulatory molecules CD40 (3/23), CD80 (B7-1, 1G10), and CD86 (B7-2, GL1).

**DC assays**

To measure DC Ag uptake, we injected mice with 0.2 mg of FITC-conjugated OVA (Sigma-Aldrich) via the tail vein. The mice were sacrificed after 1 h and FACS sorted the cells to obtain myeloid DCs. We injected OVA (Sigma-Aldrich) via the tail vein. The mice were sacrificed after 1 h and FACS sorted the cells to obtain myeloid DCs. We next characterized the composition of the liver DCs expanded after BDL. By day 3, we identified a dramatic shift toward the myeloid subset of liver DCs (Fig. 2A). Backgating analysis showed that this enriched proportion of myeloid DCs was evenly distributed among the highly granular population of cells seen on flow cytometric scatter plots (not shown). More than 60% of the DCs in mice treated with BDL were myeloid compared with untreated or sham-operated controls. At day 7 after BDL 70% of DCs were myeloid, and by day 14 the percentage was >80%. As a result of the increased overall number of DCs and the higher proportion of myeloid DCs, BDL resulted in a >15-fold increase in the total number of myeloid DCs (Fig. 2B). The effects of BDL on DCs were specific to the liver, as the relative composition of splenic DC subsets was unchanged.

**Results**

**Bile duct obstruction increases the number of liver DCs**

To ascertain the effect of obstructive jaundice on hepatic dendritic cells, we first assessed whether there were changes in the number of hepatic NPCs. Using our previously established method using CD45 immunomagnetic beads to exclude CD45<sup>-</sup> liver sinusoidal endothelial cells (19), we found that 3, 7, and 14 days after BDL there were considerably more CD45<sup>+</sup> NPCs in BDL-treated mice compared with untreated or sham-operated mice (Table I). We then used flow cytometry to determine the composition of the expanded CD45<sup>+</sup> NPC population. Based on forward and side light scatter, it was clear that a unique population of large, granular cells developed in mice treated with BDL (Fig. 1). This population comprised ~70% of the CD45<sup>+</sup> NPCs in the liver but was essentially absent in the spleen. On further analysis, we found that this new population contained ~45% CD11c<sup>+</sup>Gr-1<sup>+</sup> cells. In addition, the CD11c<sup>+</sup> DC fraction increased from ~10% in controls to 15% in mice subjected to BDL 3 days earlier. By days 7 and 14, the percentage of DCs was as high as 19% (Table I). The increase in the proportion of DCs was specific to the liver and was not found in the spleen. Taken together, the increased number of NPCs and the higher percentage of CD11c<sup>+</sup> DCs after BDL resulted in an ~4-fold increase in the total number of hepatic DCs by day 14.

**BDL selectively expands myeloid DCs within the liver**

We next characterized the composition of the liver DCs expanded after BDL. By day 3, we identified a dramatic shift toward the myeloid (CD11c<sup>+</sup>CD11b<sup>+</sup>CD8α<sup>+</sup>) subset of liver DCs (Fig. 2A). Backgating analysis showed that this enriched proportion of myeloid DCs was evenly distributed among the highly granular population of cells seen on flow cytometric scatter plots (not shown). More than 60% of the DCs in mice treated with BDL were myeloid compared with ~20% in untreated and sham-operated controls. At day 7 after BDL 70% of DCs were myeloid, and by day 14 the percentage was >80%. As a result of the increased overall number of DCs and the higher proportion of myeloid DCs, BDL resulted in a >15-fold increase in the total number of myeloid DCs (Fig. 2B). The effects of BDL on DCs were specific to the liver, as the relative composition of splenic DC subsets was unchanged.

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<th>Table I. Bile duct ligation increases the number of NPCs and the proportion of bulk liver DCs&lt;sup&gt;a&lt;/sup&gt;</th>
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<td><strong>Day 3</strong></td>
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<sup>a</sup> Three to four mice per group per time point were analyzed individually. Data presented are ± SEM.

<sup>b</sup> CD45<sup>+</sup> NPCs or splenocytes × 10<sup>7</sup>.

<sup>c</sup> *, p < 0.05 (determined using the two-sample t test).
Liver myeloid DCs in BDL have enhanced function

Although there was a striking increase in the number of myeloid DCs following BDL, our original hypothesis was that liver DC function would be impaired. To test this idea, we studied the function of the expanded liver myeloid DCs. We first analyzed Ag uptake in vivo. We administered FITC-OVA i.v. to mice 3 days after BDL. We harvested the liver 30 min later and measured DC fluorescence. Both bulk (not shown) and liver myeloid DCs (Fig. 3A) from BDL-treated mice captured considerably more Ag on a per cell basis than DCs from either untreated or sham-operated controls.

We next tested the ability of DCs from mice treated with BDL to stimulate T cells, the hallmark function of DCs. In a MLR we found that bulk DCs from mice subjected to BDL were more effective at stimulating allogeneic T cells to divide (Fig. 3B). Using cell sorting to isolate myeloid DCs, we found that they induced greater allogeneic proliferation at all time points (days 3, 7, and 14) tested (Fig. 3C). There was some nonspecific enhancement of DC allostimulatory ability from sham laparotomy, but this effect was only present for 1 wk, whereas myeloid DCs from BDL mice retained enhanced function at 2 wk. To assess whether myeloid DCs also had increased ability to stimulate Ag-specific T cells, we used TCR-transgenic T cells. Liver myeloid DCs from day 3 BDL mice produced markedly higher stimulation of H-2Kb-restricted Ag-specific CD4+ T cells when loaded with OVA323–327 (Fig. 3D). In contrast, splenic myeloid DC function was unchanged (Fig. 3E).

LPS further enhances the function of liver myeloid DCs from BDL-treated mice

We next wanted to determine whether the increased function of the expanded myeloid DCs merely resulted from an increase in maturation status, because mature DCs have enhanced T cell stimulatory ability. BDL did not alter the maturation markers MHC class II, CD40, CD80, and CD86 as compared with DCs from controls (Fig. 4). Furthermore, myeloid DCs from mice treated with BDL did not have less apoptosis or greater in vitro survival than controls as measured by 7-aminoactinomycin D and annexin staining (not shown).

To understand the increased function of expanded myeloid DCs, we then examined their cytokine profile. Liver myeloid DCs from sham or BDL mice treated 3 days previously made barely detectable amounts of IL-6, IL-10, IL-12, TNF-α, and IFN-γ in vitro. However, because BDL may be associated with increased exposure to endotoxin (3), we tested the in vitro cytokine response to LPS. We found that whereas liver myeloid DCs from untreated and sham controls made small amounts of IL-6, those from BDL mice made almost 900 pg/ml (Fig. 5A). To what extent liver myeloid DCs contribute to elevated serum IL-6 levels after BDL, which are reported to be up to 50 pg/ml (20, 21), was not determined. There were no significant differences from controls in the amounts of IL-10, IL-12, TNF-α, or IFN-γ made in response to LPS (not shown).

Given the effect of LPS on cytokine production, we performed additional MLRs in the presence of LPS to test the effect on APC function. We found that LPS increased the allogeneic proliferation in all groups (Fig. 5B), which was consistent with our previous findings showing that IL-6 increases the allosstimulation of both bone marrow and freshly isolated splenic DCs (22). Notably, though, liver myeloid DCs from BDL-treated mice remained significantly more stimulatory.
Liver myeloid DC expansion depends on Gr-1+ cells

Because it is well established that liver injury increases systemic levels of both circulating IL-6 and TNF-α (21, 23), we also tested their contribution to liver myeloid DC expansion after BDL. First, we attempted to recapitulate the in vivo milieu, thinking that perhaps resident DCs or other NPCs might be induced to become myeloid DCs. We cultured liver NPC or CD11c CD11b CD8α~ hepatic DCs, which we have described previously (13), in IL-6 (10 ng/ml), TNF-α (20 ng/ml), or both. We could not demonstrate an increase in myeloid DCs in comparison to wells with medium alone (not shown). We next performed BDL in both IL-6−/− and TNF-α−/− mice. We found a nearly identical myeloid shift as in wild-type mice, demonstrating that elevated circulating levels of IL-6 or TNF-α alone were not responsible for myeloid DC expansion in the liver following BDL (Fig. 6A).

Because we had found an influx of Gr-1+ cells in the liver of mice treated with BDL, we postulated that this cell population might be responsible for recruiting myeloid DCs. To test this hypothesis, we isolated highly purified CD45+ NPCs with the Ova323-339 peptide and then isolating myeloid DCs by FACS and incubating them with 5×10^4 Ova323-339 TCR-transgenic T cells. OT-II T cells alone had minimal (~80 cpm) proliferation. E. MLRs were performed using sorted spleen myeloid DCs. Unless specified, results are shown for day 3 after BDL or sham operation. Data are averages from duplicate or triplicate wells and presented as mean ± SEM.

**FIGURE 3.** BDL increases the function of liver myeloid DCs. A. Ag uptake assays were performed by injecting 0.2 mg of FITC-OVA via the tail vein 3 days after BDL. CD45+ NPCs were isolated 30 min later, and myeloid (CD11c~ CD11b~ CD8α~) DCs were analyzed with flow cytometry. Data shown are an average of two separate samples per group and are representative of two separate experiments. B. An MLR was performed by mixing various numbers of CD11c immunomagnetic bead-purified, bulk liver DCs with 2×10^5 allogeneic (BALB/c) T cells. T cells incubated alone had minimal (~440 cpm) proliferation. C. An MLR was performed by mixing sorted liver myeloid DCs isolated with 1–3×10^5 allogeneic (BALB/c) T cells. The overall higher levels of proliferation seen on day 14 were due to higher numbers of T cells per well. Data shown are representative of three separate experiments. D. An MLR was performed by mixing CD45+ T cell activation was tested by loading CD45+ NPCs with the Ova323-339 peptide and then isolating myeloid DCs by FACS and incubating them with 5×10^4 Ova323-339 TCR-transgenic T cells. OT-II T cells alone had minimal (~80 cpm) proliferation.
mice with an anti-Gr-1 Ab and then subjected them to BDL. By day 3, we found up to a 70% reduction in the recruitment of liver myeloid DCs (Fig. 6C). Direct depletion of myeloid DCs by the anti-Gr-1 Ab could not have been responsible for this finding, because only 10–15% of myeloid DCs bore this marker (not shown). Using immunofluorescence, we demonstrated that, following BDL, granulocytes and DCs were increased in the liver and that they colocalized near intrahepatic vessels (Fig. 6D).

Discussion
The immunologic effects of obstructive jaundice are complex and incompletely understood. Because dendritic cells are now known to be major regulators of immunity, we postulated that immunosuppression in jaundice may be partially explained by alterations in liver dendritic cells. However, we found that BDL not only expanded the number of liver DCs but also increased their function. Strikingly, liver DC composition became skewed to a myeloid phenotype. In fact, ~80% of liver DCs became myeloid by day 14 after BDL compared with a baseline of 20%, and the absolute increase in the number of liver myeloid DCs was >15-fold. Although other investigators have demonstrated examples of DC recruitment in the setting of inflammatory responses both in vitro (24) and in vivo (25, 26), selective expansion of myeloid DCs has not been demonstrated other than in the setting of exogenous cytokine administration (27). We have shown previously that overexpression of GM-CSF can expand liver myeloid DCs in vivo (28). However, we did not find expansion of DC precursors following BDL as we had after GM-CSF treatment.

Myeloid DCs have been generally recognized as being more immunogenic than lymphoid or plasmacytoid DCs (29). Previously, we demonstrated that liver myeloid DCs were more potent inducers of immunity than liver lymphoid DCs (13). In the current study, we found at all of the time points tested after BDL that liver myeloid DCs induced greater proliferation of both allogeneic and syngeneic T cells than did controls. The increased function of liver myeloid DCs was not explained by changes in DC maturation and could be further increased in vitro by LPS. Our study was not designed to address the contribution of infection in obstructive jaundice but rather to determine the global effects of BDL (including cholestasis, bacterial colonization of bile, and perhaps infection) on liver DCs. Greve et al. (3) showed that the immunologic derangement of cellular immunity in jaundiced rodents was mediated by the presence of endotoxin, and bacteria is certainly present in some jaundiced patients (30). Accordingly, we found that three of four mice had bacteria in their bile and blood 3–4 days following BDL (not shown), even though there were no obvious signs of sepsis. Despite the presence of bacteremia, we found myeloid DC expansion only in the liver and not in the spleen. In addition, LPS

![FIGURE 4.](image-url) BDLS does not alter the maturation status of liver myeloid DCs. Surface expression of the maturation markers MHC II, CD40, CD80, and CD86 were similar 3 days after BDL and at 7 and 14 days (not shown). Isotypes are indicated by open histograms. Data are representative of >5 separate experiments.

![FIGURE 5.](image-url) LPS increased the function of liver myeloid DCs from BDL-treated mice. A, Sorted liver myeloid DCs from mice treated 3 days earlier were resuspended at 3.4 × 10⁶ cells/ml with LPS (1 μg/ml). After 72 h, supernatant was tested for the presence of IL-6 using a cytometric bead array. Data are presented as mean ± SEM. B, A MLR was performed using sorted liver myeloid DCs from mice treated with BDL 3 days earlier. DCs were mixed with 2 × 10⁶ allogeneic (BALB/c) T cells. In some groups, 2 μg/ml LPS was added. Results are averages from duplicate or triplicate wells. Data are presented as mean ± SEM. T cell proliferation alone was negligible.
alone was not sufficient to induce the expansion of liver myeloid DCs 3 days after an i.p. dose as high as 100 μg (not shown).

Because we identified dramatic differences in the number and function of liver myeloid DCs after BDL, we wanted to determine whether the effects were specific to liver DCs. In the spleen, there was no evidence of an “injury” population of cells that had increased size and granularity. Moreover, the total number of splenic DCs was unchanged. Although the myeloid subtype normally comprises a much larger proportion of total DCs in the spleen as compared with the liver, BDL did not increase it further. BDL also did not alter splenic DC function. These findings, along with the fact that liver myeloid DC expansion was preserved in IL-6 knockout mice, suggested that local factors may be involved. Chiu et al. (31) implicated CCR2 ligands in the function of lung DCs in an experimental model of inflammation. These investigators showed that although recruitment was unaffected in CCR2 knockout mice, lung DCs were less mature and elaborated lower amounts of cytokines in response to activation. Sidhu et al. (32) showed that neutrophils were a critical mediator in the influx of Ag-nonspecific lymphocytes into the liver, consistent with our finding that Gr-1+ cells were critical for liver myeloid DC expansion. However, despite screening a large number of chemokines, they were not able to identify the mechanism further. Obstructive jaundice is known to induce rapid accumulation of granulocytes into the liver. The accumulation is thought to depend on cytokine-induced neutrophil chemoattractant (33). MCP-1 is a chemokine that is known to attract DCs (24–26). Although we identified Gr-1+ cells as critical to the recruitment of liver myeloid DCs, we further implicated MCP-1 as a potential mediator. In mice subjected to BDL, the CD11c− fraction of CD45+ NPCs (which is largely comprised of Gr-1+ cells) made high levels of MCP-1. Granulocytes also colocalized with DCs by immunofluorescence analysis of livers from BDL-treated mice.

The function of liver DCs is just beginning to be unraveled. We and three other groups have characterized liver DC subsets in mice. Using CD11b and CD8α we defined four DC subsets, including myeloid (CD11b+CD8α−) and lymphoid (CD11b−CD8α+) DCs, DCs with intermediate expression for both markers (CD11blow/CD8alow), and DCs lacking both lineage markers (CD11b−CD8α−). In addition, we identified a significant population of plasmacytoid DCs characterized by B220 expression (CD11c−B220+). Lian et al. (15) characterized a group of myeloid and plasmacytoid liver DCs based on B220 and CD11b expression but did not detect a lymphoid DC subset that expresses CD8α and DEC205. We found that bulk liver DCs had less T cell stimulatory ability than bulk splenic DCs, but the liver myeloid and lymphoid subtypes had equivalent function compared with their splenic counterparts. Meanwhile, Johannsen and Wick (17) reported liver DCs based on their expression of CD11c and CD8α and described heterogeneous expression of CD11b between those two groups; but they did not identify a B220+ DC population. Lastly, in
the report of Jomantaite et al. (14), liver DCs were categorized based on the relative expression of CD8α and CD11b initially, but for functional analysis the myeloid and lymphoid fractions were combined as a CD11c+/intermediate B220− group. In their analysis, the hepatic DCs were divided into two groups based solely on the expression of B220, and, consistent with our findings, they showed that bulk B220− splenic DCs were more stimulatory. It is possible that the function of other hepatic DC subsets is also affected by BDL; however, there were simply too few of these cells for analysis.

Thus, BDL alters the subset composition of liver DCs and increases liver DC function. Recent focus on the mechanisms underlyng hepatic fibrosis have found that activation of the hepatic mesenchymal cells plays a major role and that lymphocyte stimulation is one of the possible pathways for the activation (34). Chronic inflammatory states and immune dysregulation play a role in other diseases that result in hepatic fibrosis, such as autoantibodies in primary biliary cirrhosis (35). Our surprising findings of increased immune function may have significant implications for further clarifying the mechanism of hepatic inflammation and fibrosis that occurs in obstructive jaundice.

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Disclosures

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