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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.

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) mice 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.

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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+ and CD8α+ 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 unstimulated cells, and compensation was set using single-stained positive controls for each color. We stained $1 \times 10^6$ 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-FcγRII/III Ab (clone 2.4G2; mAb Core Facility, Sloan-Kettering Institute). In some cases dead cells, which typically comprised 3–7%, were selected and CD45- hepatic NPCs, were excluded with either 7-aminoactinomycin D (BD Biosciences) or 4,6-diamidino-2-phenylindole, dilactate. Slides were mounted using ProLong Gold antifade reagent (Invitrogen Life Technologies). Pictures were taken with a confocal laser-scanning microscope (Zeiss LSM 510).

Cytokine measurement

Cytokine production was measured after 36–48 h of culture. In some cases, LPS (1 μg/ml; Sigma-Aldrich) was added to the culture medium. Supernatant was harvested from triplicate wells, and IL-6 and MCP-1 levels were measured using a cytometric bead array (BD Biosciences).

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 involving CD45 immunomagnetic beads to exclude CD45− liver sinusoidal endothelial cells (19), we found that 3, 7, and 14 days after BDL there were considerably more CD45+ 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+ 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+ NPCs in the liver but was essentially absent in the spleen. On further analysis, we found that this new population contained ~45% CD11c− 11b−Gr−1+ cells. In addition, the CD11c− 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+ DCs after BDL resulted in a ~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−CD11b+CD8α−) 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 numbers 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.

Table I. Bile duct ligation increases the number of NPCs and the proportion of bulk liver DCs

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<tr>
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<th>Day 3 Cells</th>
<th>Day 7 Cells</th>
<th>Day 14 Cells</th>
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<tr>
<td></td>
<td>DC (%)</td>
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<td>Liver</td>
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<td>Untreated</td>
<td>4.7 ± 0.2</td>
<td>5.1 ± 1.3</td>
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<tr>
<td>Sham</td>
<td>4.2 ± 0.6</td>
<td>8.6 ± 0.4</td>
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<tr>
<td>BDL</td>
<td>9.8 ± 2.0</td>
<td>14.9 ± 0.6*</td>
<td>7.0 ± 1.7</td>
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<td>Spleen</td>
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<tr>
<td>Untreated</td>
<td>117.0 ± 10.0</td>
<td>1.1 ± 0.1</td>
<td>90.5 ± 5.0</td>
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<tr>
<td>Sham</td>
<td>111.0 ± 8.0</td>
<td>1.4 ± 0.2</td>
<td>150.0 ± 5.3</td>
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<tr>
<td>BDL</td>
<td>91.0 ± 5.0</td>
<td>0.8 ± 0.2</td>
<td>159.0 ± 23.0</td>
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</table>

* Three to four mice per group per time point were analyzed individually. Data presented are ± SEM.
* CD45− NPCs or splenocytes × 104.
* *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 liver 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 allogeneicity in all groups (Fig. 5B), which was consistent with our previous findings showing that IL-6 decreases the allostimulation 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<sup>+</sup> 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<sup>-</sup>CD11b<sup>-</sup>CD8α<sup>-</sup> 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<sup>-/-</sup> and TNF-α<sup>-/-</sup> 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<sup>+</sup> 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<sup>-</sup>CD11c<sup>+</sup> NPCs and measured their secretion of a panel of cytokines. We found that CD11c<sup>+</sup> NPCs from mice that had undergone BDL 3 days earlier made high levels (13 ng/ml) of MCP-1 compared with untreated and sham-operated controls (Fig. 6B). To prove that Gr-1<sup>+</sup> cells were responsible for myeloid DC expansion in vivo, we depleted mice, depleting mice, depleting 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).

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**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<sup>+</sup> NPCs were isolated 30 min later, and myeloid (CD11c<sup>-</sup>CD11b<sup>-</sup>CD8α<sup>-</sup>) 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<sup>5</sup> 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<sup>5</sup> 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, Ag-specific CD4<sup>+</sup> T cell activation was tested by loading CD45<sup>+</sup> NPCs with the Ova<sub>323–339</sub> peptide and then isolating myeloid DCs by FACS and incubating them with 5 × 10<sup>4</sup>Ova<sub>323–339</sub> 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.
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. 6 C). Direct depletion of myeloid DCs by the
anti-Gr-1 Ab could not have been responsible for this finding, be-
cause 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. 6 D).

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 re-
sponses both in vitro (24) and in vivo (25, 26), selective ex-
pansion 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 ex-
pand 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). Previ-
ously, 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 (includ-
ing cholestasis, bacterial colonization of bile, and perhaps infect-
on) on liver DCs. Greve et al. (3) showed that the immunologic
derangement of cellular immunity in jaundiced rodents was mediat-
ed 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 follow-
ing 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 5. 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 × 10^5
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 × 10^5 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 and TNF- 

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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/H9251* 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 underlying hepatic fibrosis have found that activation of the hepatic mesenchymal cells plays a major role and that lymphocytic 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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