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NOD2 Ligation Subverts IFN-α Production by Liver Plasmacytoid Dendritic Cells and Inhibits Their T Cell Allostimulatory Activity via B7-H1 Up-Regulation

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The nucleotide-binding oligomerization domain (NOD)2/CARD15 protein, which senses muramyl dipeptide (MDP), a product of bacterial peptidoglycan, appears to play an important role in regulating intestinal immunity. Although the liver is exposed to gut-derived MDP, the influence of NOD2 ligation on hepatic APC, in particular dendritic cells (DC), is unknown. Freshly isolated mouse liver and spleen plasmacytoid (p)DC expressed higher levels of NOD2 message than conventional myeloid (m)DC. Following MDP stimulation in vivo, liver pDC, but not mDC, up-regulated expression of IFN regulatory factor 4 (IRF-4), a negative regulator of TLR signaling, and induced less allogeneic T cell proliferation and IFN-γ production. The adoptive transfer of liver pDC from MDP-treated mice failed to prime allogeneic T cells in vivo. By contrast, splenic DC IRF-4 levels and T cell stimulatory activity remained unchanged. Liver pDC from MDP-stimulated mice also displayed greater IκBα, cell surface B7-H1, and B7-H1 relative to CD86 than control liver pDC. No similar effects were observed for liver mDC or spleen DC. Absence of B7-H1 on liver pDC reversed the inhibitory effect of MDP. After ex vivo stimulation with LPS or CpG, liver pDC but not mDC from MDP-treated animals secreted less IL-12p70, IL-6, and TNF-α and induced weaker allogeneic T cell proliferation than those from controls. Moreover, CpG-stimulated liver pDC from MDP-treated mice secreted less IFN-α than their splenic counterparts, and systemic levels of IFN-α were reduced in MDP-treated animals after CpG administration. These findings suggest that differential effects of NOD2 ligation on liver pDC may play a role in regulating hepatic innate and adaptive immunity.

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§ Abbreviations used in this paper: DC, dendritic cell; CARD, caspase activation and recruitment domain; CM, culture medium; Fugu-like tyrosine kinase 3 ligand; IRF, IFN regulatory factor; mDC, myeloid DC; MDP, muramyl dipeptide; MFI, mean fluorescence intensity; mPDCA, mouse pDC Ag; ND, not detectable; NOD, nucleotide-binding oligomerization domain; pDC, plasmacytoid DC; TRAF, TNFR-associated factor; Treg, regulatory T cell.

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modular domain organization of a C-terminal leucine-rich repeat domain that binds MDP, a central NOD domain, and an N-terminal effector domain composed of a CARD, pyrin domain, or baculovirus “inhibitor of apoptosis” repeat (Bir) domain (24). Upon sensing MDP, NOD2 undergoes conformational changes that lead to a CARD-CARD interaction with the receptor-interacting serine/threonine kinase RICK, a downstream effector molecule that mediates ubiquitination of IκB kinase-γ (IKK-γ), which is associated with NF-κB activation and transcription of proinflammatory cytokines (23).

The functional significance of NOD2 in the regulation of immune responses is underscored by the fact that a subpopulation of Crohn’s disease patients bears mutations in the NOD2 gene (25–26) that are associated with a younger age at diagnosis, ileal involvement, ileocecal resections, and a high risk of postoperative relapse and reoperation (27). Moreover, there is recent evidence that systemic administration of MDP prevents the development of experimental colitis in mice through the suppression of multiple TLR pathways (TLR2, TLR4, and TLR9) with an associated reduction in Th1 responses (28). Hedl et al. (29) have reported that, unlike peripherally derived macrophages, intestinal macrophages secrete TNF-α after acute MDP stimulation, suggesting a possible ‘‘MDP tolerance’’ mechanism similar to that described for LPS. Furthermore, prolonged exposure of macrophages to MDP reduces proinflammatory cytokine production after rechallenge with TLR ligands (“cross-tolerance”) (29). These regulatory effects of NOD2 signaling have recently been correlated with the induction of a gene transcription regulatory protein, IFN regulatory factor (IRF) 4 (28), that negatively regulates TLR signaling (30, 31). Taken together, these findings suggest an important role of NOD2 signaling in regulating innate and adaptive immunity.

The liver is exposed continually to MDP, yet no data are available concerning the expression and role of NOD2 in liver DC subsets. In this study, we aimed to analyze NOD2 expression in liver mDC and pDC to ascertain the influence of in vivo NOD2 ligation on liver DC function and to address mechanisms underlying the observed effects.

Materials and Methods
Experimental animals
Male C57BL/10 (B10) (H-2b) and BALB/c (H2d) mice (8-12 wk old) were purchased from The Jackson Laboratory. B7-H1-deficient mice (B6[H-2b] background) were described previously (32). They were maintained in the specific pathogen-free facility of the University of Pittsburgh School of Medicine (Pittsburgh, PA). Experiments were conducted in accordance with the guidelines for the care and use of laboratory animals from the National Institutes of Health and an Institutional Animal Care and Use Committee-approved protocol of the University of Pittsburgh School of Medicine. Mice received Purina rodent chow (Ralston Purina) and tap water ad libitum.

Reagents
RPMI 1640 complete medium was used for cell culture as described (19). Chinese hamster ovary cell-derived recombinant human Fms-like tyrosine kinase 3 ligand (Flt3L) was provided by Amgen (Seattle, WA). MDP and LPS (from Salmonella minnesota R595; specific for TLR4) were purchased from InvivoGen and Alexis Biochemicals, respectively. CpG oligodeoxynucleotides were purchased from InvivoGen. Isolation of liver and spleen DC subsets
CD11c+ cells were isolated from livers and spleens of mice given the endogenous DC poietin Flt3L (10 μg/mouse/day i.p. for 10 days) as described (16) and either MDP (100 μg/mouse/day i.p.) or PBS for the last 3 days of cytokine treatment. Bulk DC were enriched by density centrifugation using Nyoczen (Sigma-Aldrich). For pDC purification (>95%) mPDCA1+ cells were positively selected from the DC-enriched fraction using immunomagnetic beads and a paramagnetic LS column (Miltenyi Biotec). For mDC purification, mPDCA1+ cells collected in the negative fraction after pDC selection were first run through LD columns (Miltenyi Biotec) to remove residual mPDCA1+ cells, then incubated with anti-mouse CD11c immunomagnetic beads (clone N418; Miltenyi Biotec), and positively selected by passage through a paramagnetic LS column, yielding a highly enriched (≥95%) CD11c+ mPDCA1+ population.

Flow cytometry (cell surface staining)
Cells were treated with Fcy-R-blocking rat anti-mouse CD16/32 mAb (2.4G2) to avoid nonspecific Ab binding. For cell surface staining, they were then incubated for 30 min with FITC-, PE-, PE-Cy5-, or PE-Cy7-conjugated mAbs to detect expression of CD11c (HL3), B220 (CD45R) (RA3-6B2), Ly6G (2B5-9-17), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), or B7-H1 (MIH5) (eBioscience). All mAbs and appropriate Ig isotype controls were obtained from BD Pharmingen unless specified otherwise. Flow analysis was performed using a LSR II flow cytometer (BD Biosciences) and results were expressed as the percentages of positive cells and mean fluorescence intensity (MFI).

RT-PCR
RNA was extracted using TRIzol (Invitrogen) and then reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Reactions for semiquantitative PCR were performed with Fast SYBR Green PCR master mix (Applied Biosystems) and primers specific for NOD2 (forward: 5′-GCTG CCAAATGCTTACGTCGTC-3′; reverse: 5′-TAAGTACTCAGGAGAACGGA GACTGTA-3′), IRF-4 (forward: 5′-GCCCAACAGCTGAAGAAG-3′; reverse: 5′-TCTCTGGAGGGTGCTGAAACT-3′) (31), IFN-α4 (from SABiosciences), or β-actin (forward: 5′-AGAGGGAAATCTGGTGTCGTC-3′; reverse: 5′-CAATAGTGATGAGCTGCGCT-3′) in triplicate. Reactions were amplified with an Applied Biosystems PRISM 7500 Fast real-time PCR system at an annealing temperature of 62°C. Data were plotted using the manufacturer’s software as the ΔΔct cycle number at which the Δct crosses this threshold. Relative gene expression was determined by extrapolation from a standard curve amplified from control cDNA and then normalized to the expression of β-actin mRNA using the comparative cycle threshold method.

Western blot analysis
Cytoplasmic and nuclear proteins were resolved in 10% SDS-polyacryl- amide electrophoresis gels, electrotransferred to nitrocellulose, and subjected to Western blot analysis. IκBα Abs were purchased from Cell Sig- naling. Western blotting signals were obtained using commercially available HRP-conjugated secondary Abs and developed using the Super-Signal chemiluminescent substrate purchased from Pierce.

T cell purification
Bulk T cells were purified from spleens and lymph nodes of normal BALB/c mice. Single cell suspensions were incubated with a mAb mixture consisting of anti-CD45R/B220 (RA3-6B2), anti-CD16/CD32 (2.4G2), anti-CD3-19, and anti-Ly6G (RB6-8C5) obtained from BD Pharmingen. After washing, non-T cells were eliminated from the cell suspension by adding mouse depletion Dynabeads (Dynal Biotech) and following the manufacturer’s instructions.

Mixed leuкоcyte reaction
Graded numbers of B10 DC were used as stimulators of normal bulk allogeneic BALB/c T cells (2 × 104/well) in a 72-h MLR using 96-well, round-bottom plates as described (33). For the final 16–18 h, individual wells were pulse-labeled with 1 μCi of [3H]thymidine. Radioisotope incor- poration was determined using a beta scintillation counter and results were expressed as mean cpm ± 1 SD of triplicate wells.

Adaptive cell transfer
Flt3L-expanded CD11c+ mPDCA1+ pDC were isolated from livers and spleens of PBS- or MDP-treated B10 mice using immunomagnetic beads (Miltenyi Biotec). Two million cells were adaptively transferred by i.v. injection (lateral tail vein) into normal, allogeneic recipients (BALB/c). Seven days later, proliferative responses of host T cells (2.104) to stimu- lation with various numbers of donor CD3+ splenocytes were determined by thymidine incorporation in MLR, as described above.
Intracellular cytokine staining

Purified DC were treated with brefeldin A (1 μl/ml; GolgiPlug, BD Pharmingen) for 16 h, labeled with FITC-conjugated anti-CD11c (HL3) and PE-Cy5-conjugated anti-B220/CD45R (RA3-6B2) mAb, and then fixed in 2% (v/v) paraformaldehyde. The cells were permeabilized with 0.1% saponin and then incubated with PE-conjugated anti-IL-12p40/p70 (C15.6) or rat IgG (all BD Pharmingen) for 30 min. Flow analysis was performed as described above and results were expressed as percentages of positive cells.

ELISA

Levels of IL-12p70, IL-6, TNF-α, IFN-α, and IFN-γ in culture supernatants were determined by ELISA using commercial kits from BioLegend (IL-12p70, IL-6, TNF-α, and IFN-γ) and PBL Biomedical Laboratories (IFN-α) respectively, following the manufacturers’ instructions. The limits of detection for IL-12p70, IL-6, TNF-α, IFN-α and IFN-γ were 7.8, 2.0, 2.0, 12.5 and 15.6 pg/ml, respectively.

Statistical analysis

Data are expressed as means ± 1 SD. Significance of differences between means was determined by an unpaired Student’s t test. A value of p < 0.05 was considered significant.

Results

Freshly isolated liver and spleen DC express NOD2 and pDC express higher levels than mDC; in vivo treatment with MDP does not change the number and relative proportion of liver and spleen DC subsets

Under physiological conditions, NOD2 is expressed mainly by epithelial cells and APC (34), including macrophages and bone marrow-derived DC, as well as human blood monocytes, DC, and monocyte-derived DC (35, 36), but not by other hematopoietic cells (37, 38). No data are available concerning NOD2 expression by liver or spleen DC subsets. To address this question, freshly isolated, immunobead-purified liver and spleen pDC (CD11c<sup>+</sup>mPDCA1<sup>+</sup>B220<sup>+</sup>) and mDC (CD11c<sup>-</sup>mPDCA1<sup>-</sup>B220<sup>-</sup>) from Flt3L-treated B10 mice (purity > 95%; Fig. 1A) were analyzed for NOD2 mRNA expression by semi-quantitative RT-PCR as described in Materials and Methods. As shown in Fig. 1A, both liver and spleen DC subsets expressed NOD2 mRNA. In each tissue, 2- to 3-fold higher levels of mRNA were expressed in pDC compared with mDC. We next analyzed the absolute number of in vivo, Flt3L-expanded, immunobead-purified liver and spleen DC subsets isolated from PBS- and MDP-treated mice at a dosage (100 μg/day for 3 days i.p.) shown by Watanabe et al. (28) to prevent experimental colitis in mice. As shown in Fig. 1C, no differences in absolute DC numbers were detected between the PBS and MDP groups. The data confirmed in both groups the relative abundance of pDC in the liver compared with the spleen.

Freshly isolated liver pDC from MDP-treated animals exhibit impaired ability to induce allogeneic T cell proliferation and IFN-γ production

Ag presentation, T cell activation/proliferation, and IFN-γ secretion are pivotal steps in inducing and regulating immune responses. In the next series of experiments, we tested whether MDP infusion of mice could modify the T cell stimulatory function of DC subsets. To address this issue, Flt3L-expanded, freshly isolated liver or spleen pDC or mDC from MDP- or PBS-injected (control) B10 mice were cocultured in MLR with normal allogeneic (BALB/c) bulk T cells for 72 h. T cell proliferation and IFN-γ levels in culture supernatants were determined by MLR and ELISA as described in Materials and Methods. As shown in Fig. 2A, liver pDC isolated from MDP-treated animals exhibited much inferior T cell allostimulatory activity when compared with those from control mice. By contrast, liver mDC from MDP-treated animals showed no significant change in their ability to induce T cell proliferation. On the other hand, the T cell stimulatory activity of either spleen pDC or spleen mDC from MDP-injected animals was not affected (Fig. 2, A and B). Although liver pDC pre-exposed to MDP in vivo showed significantly impaired ability to induce IFN-γ secretion in MLR (Fig. 2C), no significant changes in IFN-γ production were detected when liver mDC or spleen DC subsets were used as T cell stimulators in MLR (Fig. 2C and data not shown).

Adoptive transfer of liver pDC from MDP-treated animals fails to prime allogeneic T cells in vivo

We next examined the ability of liver and spleen pDC from control or MDP-treated B10 mice to prime normal allogeneic T cells in vivo. As shown in Fig. 3, T cells harvested from BALB/c mice
DC subsets from MDP- or PBS-treated animals. As shown in Fig. 4B, molecules on Flt3L-expanded, freshly isolated liver and spleen coinhibitory B7-H1 (also called PD-L1) and costimulatory CD86 family molecules, we next examined the cell surface expression of depend on the net coregulatory signals delivered by DC-expressed B7. Because DC function and the outcome of DC-T cell interactions may depends on pretreatment with MDP resulted in complete suppression of the effect of MDP on liver pDC, Flt3L-expanded, freshly isolated, immunobead-purified liver and spleen pDC and mDC from MDP-treated (100 μg/day for 3 days i.p.) or PBS-treated B10 mice were used as stimulators in 72 h MLR (responder cells were BALB/c bulk T cells). A, T cell proliferation was quantified by [3H]Tdr incorporation. The proliferation of BALB/c T cells cultured alone (Neg (negative) control) and the responses of T cells stimulated with pDC or mDC isolated from CpG-ODN B (CpG)- or LPS-treated (LPS) mice (positive controls) are also shown. Data are representative of three independent experiments, with two mice per group in each experiment; * p < 0.004. B, Overall statistical analysis of three independent experiments showing the percentage of T cell proliferation induced by MDP-treated liver and spleen DC subsets relative to PBS-treated groups; * p < 0.004. C, Supernatants from cocultures of liver or spleen DC subsets from MDP- or PBS-treated B10 mice with BALB/c bulk T cells were harvested at 72 h and IFN-γ levels were quantified by ELISA. Data are representative of three independent experiments, with two mice per group in each experiment; * p < 0.007 compared with MDP.

FIGURE 2. Freshly isolated liver pDC from MDP-treated animals exhibit comparatively weak ability to induce allogeneic T cell proliferation and IFN-γ production. Flt3L-expanded, freshly isolated, immunobead-purified liver and spleen pDC and mDC from MDP-treated (100 μg/day for 3 days i.p.) or PBS-treated B10 mice were used as stimulators in 72 h MLR experiment; **p < 0.004. A, T cell proliferation was quantified by [3H]Tdr incorporation. The proliferation of BALB/c T cells cultured alone (Neg (negative) control) and the responses of T cells stimulated with pDC or mDC isolated from CpG-ODN B (CpG)- or LPS-treated (LPS) mice (positive controls) are also shown. Data are representative of three independent experiments, with two mice per group in each experiment; * p < 0.004. B, Overall statistical analysis of three independent experiments showing the percentage of T cell proliferation induced by MDP-treated liver and spleen DC subsets relative to PBS-treated groups; * p < 0.004. C, Supernatants from cocultures of liver or spleen DC subsets from MDP- or PBS-treated B10 mice with BALB/c bulk T cells were harvested at 72 h and IFN-γ levels were quantified by ELISA. Data are representative of three independent experiments, with two mice per group in each experiment; * p < 0.007 compared with MDP.

FIGURE 3. Adoptive transfer of liver pDC from MDP-treated animals fails to prime allogeneic T cells in vivo. Flt3L-expanded liver or spleen pDC (2 × 10⁶) from PBS- or MDP-treated B10 mice were injected i.v. into groups of two or three normal, allogeneic (BALB/c) recipients. Seven days later, proliferative responses of host bulk T cells (2 × 10⁵) to stimulation with various numbers of B10 CD3⁺ splenocytes were determined as described in Materials and Methods. For control purposes, proliferation of unprimed BALB/c bulk T cells stimulated by B10 CD3⁺ splenocytes (Control) was tested. Results are means ± 1 SD of triplicate cultures and are from one experiment representative of two performed; *, p < 0.01.

Injected systemically 7 days previously with 2 × 10⁶ splenic pDC showed marked enhancement of anti-CD86 reactivity in MLR, irrespective of donor MDP treatment. By contrast, donors pretreated with MDP resulted in complete suppression of the ability of liver pDC to prime allogeneic T cells in vivo.

Elevated B7-H1/CD86 ratio on liver pDC from MDP-treated animals correlates with impaired T cell stimulatory ability

Because DC function and the outcome of DC-T cell interactions may depend on the net coregulatory signals delivered by DC-expressed B7 family molecules, we next examined the cell surface expression of coinhibitory B7-H1 (also called PD-L1) and costimulatory CD86 (B7-2) molecules on Flt3L-expanded, freshly isolated liver and spleen DC subsets from MDP- or PBS-treated animals. As shown in Fig. 4A, B7-H1 expression was up-regulated significantly on a subset of liver pDC from MDP-treated animals compared with DC from controls (MFI: 33.1 ± 5.0 vs 22.7 ± 3.0; p < 0.02). With regard to CD86 expression on liver and spleen DC subsets, no significant differences were detected between MDP-treated and control animals (Fig. 4B). However, when the B7-H1/CD86 ratio, reported previously to negatively correlate with lymphocyte activation (39), was calculated (Fig. 4C), only liver pDC from MDP-treated animals showed a higher ratio compared with control groups (B7-H1/CD86: 1.9 ± 0.7 vs 1.4 ± 0.4; p < 0.007). This higher B7-H1/CD86 ratio on liver pDC exposed to MDP in vivo was consistent with their inferior ability to induce T cell proliferation and IFN-γ production (Fig. 2, A and C) and to prime allogeneic T cells in vivo (Fig. 3). To verify the role of B7-H1 signaling in mediating the influence of MDP on liver pDC, we next used liver pDC isolated from B7-H1−/− animals as stimulators in a 72 h MLR. As shown in Fig. 4D, the impaired ability of liver pDC from MDP-treated mice to induce allogeneic T cell proliferation was reversed when B7-H1−/− pDC were used as stimulators, further implicating B7-H1 in the inhibitory action of MDP on liver pDC function. As with conventional DC (40), T cell proliferation induced by pDC isolated from B7-H1−/− animals was higher than that induced by DC from wild-type mice both in the PBS and MDP groups.

The liver environment plays a key role in mediating the influence of MDP on hepatic pDC

To ascertain whether the liver microenvironment is involved in the effect of MDP on liver pDC, Flt3L-expanded, freshly isolated, immunobead-purified liver pDC were cultured with or without 10–100 μg/ml MDP. Twenty-four hours later B7-H1 expression, as determined by flow cytometry, was unaffected by exposure to MDP (PD-L1 MFI: 23 ± 5 (10 μg), 22 ± 4 (100 μg) vs 21 ± 5 (0 μg), and the T cell allostimulatory activity of MDP pre-exposed liver pDC did not differ from that of control cells (supplemental Fig. S1). The discrepancy between the consequences of in vivo

4 The online version of this article contains supplemental material.
and in vitro exposure to MDP supports the hypothesis that the liver environment most likely plays a key role in mediating the in vivo effect of MDP on hepatic pDC.

Inhibition of the T cell stimulatory ability of liver pDC by MDP

It has been shown recently (28) that in vivo stimulation of NOD2 with MDP is associated with the up-regulation, in CD11b<sup>+</sup> myeloid cells, of the gene transcription regulatory molecule IRF-4, a negative regulator of the signaling cascade leading to NF-κB activation. To ascertain whether the in vivo inhibitory effect of MDP on liver pDC was associated with a change in IRF-4 expression, we performed RT-PCR on Flt3L-expanded, freshly isolated liver and spleen DC subsets isolated 8 and 24 h after MDP or PBS treatment (control). As shown in Fig. 5A, at 24 h liver but not spleen pDC from MDP-treated mice expressed significantly (3-fold) higher IRF-4 mRNA than pDC from the control group, whereas no significant differences in liver and spleen mDC IRF-4 expression were detected between the MDP- and PBS-treated groups. Analysis of liver and spleen DC subsets isolated at 8 h after the last injection of MDP did not reveal any significant difference in IRF-4 expression between the groups (data not shown), suggesting that the early kinetics of IRF-4 expression were not affected.

Ubiquitination of the inhibitor IκBα is a key and common step in different NF-κB activation pathways. Therefore, we next verified, by Western blotting, whether the increased IRF-4 levels in liver pDC from MDP-treated mice were associated with modification of IκBα inhibitor levels. As shown in Fig. 5B, higher levels of IκBα were demonstrated in liver pDC from MDP-treated animals compared with those in pDC from control mice.

In vivo MDP treatment impairs TLR4- and TLR9-induced T cell stimulatory activity of liver pDC

Under normal steady-state conditions, in vitro culture of liver and spleen DC subsets, with or without LPS or CpG, up-regulates their T cell stimulatory activity to varying degrees. Moreover, it has been shown that MDP stimulation interferes with the subsequent reactivity (NF-κB activation) of human macrophages or DC in response to TLR ligands (28, 29). Therefore, we next analyzed the ability of liver and spleen DC subsets to up-regulate their T cell stimulatory function after overnight culture with or without LPS or CpG (TLR4 and TLR9 ligands, respectively). Murine pDC have been shown to express low levels of TLR4 (16, 17, 41–43). As shown in Fig. 6A, both freshly isolated liver pDC and those cultured overnight from MDP-treated animals in medium alone exhibited inferior T cell allostimulatory activity compared with those from PBS-treated control mice. This difference in stimulatory function was more marked when the corresponding pDC population was compared following overnight culture in LPS or CpG (1 μg/ml) (Fig. 6A). Similar results were obtained when lower concentrations of LPS or CpG (10 and 100 ng/ml) were used (data not shown). By contrast, liver mDC and both spleen DC subsets exposed to MDP in vivo retained their allogeneic T cell stimulatory activity compared with DC from PBS-treated mice (Fig. 6B).
IL-12 plays a critical role in the development of Th1 cells from naive CD4+ T cells (44). We next examined whether in vivo MDP administration influenced TLR4- or TLR9 ligand-mediated IL-12 production by liver and spleen DC subsets, using intracellular cytokine staining and ELISA as described in Materials and Methods. As shown in Fig. 7, A and C, liver pDC and mDC from MDP-treated mice cultured overnight without TLR ligation displayed significantly reduced IL-12 p70 production compared with cells from control mice (pDC: not detectable (ND) vs 138 ± 25 pg/ml, p < 0.01; mDC: 160 ± 50 vs 405 ± 46 pg/ml, p < 0.01). When liver DC stimulated with MDP in vivo were cultured overnight with LPS (1 µg/ml) or CpG (100 ng/ml), IL-12 p70 secretion by pDC remained lower than that of control pDC (LPS: 220 ± 37 vs 701 ± 45 pg/ml, p < 0.01; CpG: 230 ± 26 vs 421 ± 42 pg/ml, p < 0.01), whereas liver mDC exhibited similar IL-12 secretion to cells from PBS-treated animals (LPS: 937 ± 70 vs 1043 ± 45 pg/ml, NS; CpG: 170 ± 27 vs 120 ± 76 pg/ml, NS). Similar results were obtained when lower concentrations of LPS (10 and 100 ng/ml) and CpG (10 ng/ml) were used (data not shown). However, a much higher concentration of CpG (1 µg/ml) could override the influence of MDP on liver pDC (data not shown). Spleen DC subsets isolated from MDP-treated mice did not show any differences from controls in IL-12 production after overnight culture, with or without LPS or CpG (data not shown).

In vivo MDP administration reduces TLR4- and TLR9-induced IL-6 and TNF-α production by liver pDC

IL-6 and TNF-α play important roles in innate and adaptive immune responses, and pDC are important sources of these cytokines (45, 46). Therefore we next analyzed the impact of in vivo MDP treatment on IL-6 and TNF-α production by liver pDC. Flt3L-mobilized, freshly isolated, immuno bead-purified liver pDC from MDP- or PBS-treated mice were cultured overnight with or without LPS or CpG (1 µg/ml), and levels of secreted IL-6 and TNF-α were measured by ELISA as described in Materials and Methods. As shown in Fig. 8, after culture in CM alone, no differences were detected between pDC from MDP- and PBS-treated animals (IL-6: 37 ± 11 vs 50 ± 10 pg/ml, NS; TNF-α: ND vs ND). After overnight culture with LPS or CpG, liver pDC from MDP-treated mice produced less IL-6 and TNF-α as compared with control groups (IL-6/LPS: 144 ± 15 vs 500 ± 55 pg/ml, p < 0.001; IL-6/CpG: 618 ± 24 vs 2006 ± 79 pg/ml, p < 0.001; TNF-α/LPS: 28 ± 11 vs 522 ± 25 pg/ml, p < 0.0004; TNF-α/CpG: ND vs 50 ± 7 pg/ml, p < 0.001). Similar results were obtained when lower concentrations of LPS and CpG (10 and 100 ng/ml) were used (data not shown).

Systemic MDP administration impairs TLR9-induced IFN-α production by liver pDC

Considering the higher proportion of pDC in liver than in spleen (5) and the unique ability of pDC to produce type-I IFNs that are important in antiviral immunity, we next analyzed the influence of MDP treatment on the ability of liver and spleen pDC to secrete IFN-α after overnight culture, with or without CpG stimulation. As shown in Fig. 9A, freshly isolated liver pDC from MDP-treated animals stimulated with CpG (1 µg/ml) secreted a much lower level of IFN-α than liver pDC from control animals (ND vs 37 ± 1 pg/ml, respectively; p < 0.0004). To determine the in vivo functional relevance of these findings, we measured IFN-α mRNA in freshly isolated liver pDC and IFN-α levels in serum from MDP-pretreated and control mice 6 h after CpG (7.5 µg/g, i.v.) administration. As shown in Fig. 9B, liver pDC from MDP-treated mice...
expressed significantly lower levels of IFN-α than those from controls. Furthermore, serum IFN-α concentrations were reduced >50% in the MDP-treated group (Fig. 9C).

Discussion

Many clinical and experimental observations support the view that the liver is a unique organ in which tolerance is favored over immunity (47–49). Thus, the liver appears to play an important role in oral (50) and portal venous tolerance (51), whereas hepatic allografts are accepted more readily than other types of organ transplant (52, 53) and can protect other grafts from the same donor against rejection (54, 55). Furthermore, hepatitis B and C virus infection is more likely to induce chronic infection of the liver than other organs (56, 57). Despite compelling evidence of hepatic tolerogenicity, the mechanisms that regulate the balance between tolerance and immunity in the liver microenvironment are still ill defined. Several findings support a pivotal role of hepatic APC, in particular DC (4, 14, 18).

Herein, we describe for the first time how NOD2 ligation in vivo can regulate innate and adaptive immune reactivity through selective functional modification of liver DC subsets. Our data show that freshly isolated liver pDC express higher levels of NOD2 message than conventional liver mDC and that similar differences are observed between spleen DC subsets. However, regardless of NOD2 expression, liver and spleen DC subsets show differences in phenotype and function when stimulated in vivo with the NOD2 ligand MDP. Administration of 100 μg of MDP for three consecutive days establishes a physiological amount equivalent to MDP levels in the colon (58) that prevent experimentally induced colitis (28). When compared with control liver pDC, freshly isolated liver pDC from MDP-treated animals showed weaker ability to induce allogeneic T cell proliferation with an associated reduction in IFN-α production, increased B7-H1 expression on a subset of cells, an elevated cell surface B7-H1/CD86 ratio, and increased expression of the NF-κB signaling inhibitory molecules IRF-4 and IκB. In vivo, liver pDC from MDP-treated mice failed to prime allogeneic T cells. By contrast, these differences were not detected for liver mDC or spleen DC subsets.

Our findings also show that MDP stimulation in vivo significantly interferes with subsequent responses of liver pDC to TLR4 or TLR9 ligation. Ex vivo stimulation with either LPS or CpG did not up-regulate the T cell stimulatory activity of liver pDC to the same extent as that of control liver pDC. In addition, MDP treatment reduced IL-12, IL-6, and TNF-α production by pDC with and without LPS or CpG stimulation. Furthermore, CpG-stimulated liver pDC secreted lower levels of IFN-α than their splenic counterparts, and systemic administration of CpG in MDP-treated mice induced lower levels of IFN-α message in liver pDC and lower systemic IFN-α levels than in control animals. Taken together, these findings demonstrate that systemic exposure to MDP markedly and selectively influences liver DC function, compared with secondary lymphoid tissue DC, and that liver pDC are more affected than liver mDC, reflecting their higher levels of
MDP receptor (NOD2) expression. These findings have significant implications for liver immunobiology given that, when compared with the spleen, higher proportions of pDC relative to conventional mDC are found in normal mouse livers or livers in which DC have been expanded by systemic administration of the DC poietin Flt3L (5, 42). Our data also show that MDP treatment does not affect the relatively high proportion of pDC relative to mDC found in the liver. Moreover, pDC are the major producers of type I IFNs in the body and are therefore important in innate antiviral responses, as well as in adaptive immunity and tolerance (13, 59). In addition, while mDC tend to prime Th1 responses, pDC can induce Th1, Th2, or regulatory T cell responses, depending on the nature of the Ag and the costimulatory and coregulatory signals delivered to T cells (13, 60, 61).

Recently, pDC have been implicated in the prolongation or indefinite survival of organ or skin allografts and the induction of alloAg-specific regulatory T cells (62–64). The impaired ability of liver pDC stimulated in vivo with MDP to induce T cell proliferation, together with decreased IFN-γ production in MLR, suggest a switch from Th1-polarizing ability, consistent with recent clinical observations. Thus, small intestine graft recipients with Crohn’s disease-associated NOD2 gene polymorphism and lack of normal NOD2 signaling are at a higher risk of graft rejection and loss, suggesting an important role of intact NOD2 signaling in the regulation of gut mucosal and alloimmune responses (65).

Our interest in B7-H1 expression and the B7-H1/CD86 ratio on the surface of liver pDC from MDP-stimulated and control mice is based on evidence that the balance between inhibitory B7-H1 and costimulatory B7-1(CD80)/B7-2(CD86) ligands regulates T cell function (39, 66). Indeed, we previously observed a much higher (5- to 6-fold) ratio of B7-H1 to CD86 on CpG-stimulated murine pDC compared with mDC, and also that a blockade of B7-H1 on pDC increased their T cell allostimulatory ability (64). In the present study, costimulatory molecule expression was not significantly different between liver and spleen DC subsets isolated from MDP-treated and control mice (data not shown). However, B7-H1 expression on a subset of cells (which may account for the effects observed) and the B7-H1/CD86 ratio were higher on liver pDC of MDP-treated animals. The significant positive correlation we observed between weak T cell allostimulatory activity and a high B7-H1/CD86 ratio on liver pDC from MDP-treated mice (compared with control cells) was confirmed using B7-H1−/− mice. Indeed, MDP treatment of B7-H1−/− mice did not impair the ability of their liver pDC to stimulate allogeneic T cell proliferation, suggesting a B7-H1-dependent mechanism underlying the effect of MDP. In the current study, the inhibitory effect of MDP on liver pDC was associated with the induction of a previously identified TLR and (likely) NOD2 inhibitory molecule, IRF-4 (28). IRF-4 has been shown to compete with IRF-5 for binding to MyD88. It

FIGURE 7. In vivo MDP stimulation reduces TLR4- and TLR9-induced IL-12 production by liver pDC. Ft3L-expanded, freshly isolated, immunobead-purified liver pDC and mDC from MDP-treated (100 μg/day for 3 days i.p.) or PBS-treated B10 mice were cultured overnight with LPS (1 μg/ml) or CpG (100 ng/ml) or CM alone. Thereafter, supernatants and cells were harvested to detect IL-12p40/70 production by ELISA (A and C) and intracellular staining by flow cytometry (B and D). The top histograms of A and C show ELISA data from one experiment representative of three independent experiments, with two mice per group in each; in the lower histograms the overall analysis of all three experiments is shown. B and D, Flow analysis results showing data of one representative experiment of three independent experiments, with two mice per group in each; *, p < 0.01.
isolated, immunobead-purified liver pDC from MDP-treated (100 µg/day for 3 days i.p.) or PBS-treated B10 mice were cultured overnight with LPS or CpG (1 µg/ml) or CM alone. Thereafter, supernatants were harvested to detect IL-6 and TNF-α production by ELISA. Data shown are representative of three independent experiments with two mice per group in each experiment; *, p < 0.001.

Several in vivo and in vitro studies suggest that chronic exposure to MDP induces “self” and “cross” tolerance. Hedl et al (29) have shown that primary, monocyte-derived macrophages, pre-exposed to MDP in vitro, fail to produce proinflammatory cytokines after restimulation with MDP or TLR4 ligation, and that intestinal macrophages, which are chronically exposed to gut-derived MDP, fail to produce proinflammatory cytokines (TNF-α) after TLR4 or MDP activation. Moreover, Watanabe et al. (28) have demonstrated that in vivo MDP treatment down-regulates TLR2-, TLR4-, and TLR9-mediated IL-12 production by colon lamina propria lymphocytes and that lymphocytes from mesenteric lymph nodes and intestinal lamina propria of MDP-treated mice secrete less IFN-γ when stimulated with anti-CD3 than those from controls. In the liver, the interaction between NOD2 and TLR signaling has not been previously investigated. Consistent with Watanabe et al. (28), our data demonstrate that in vivo MDP stimulation results in a down-regulatory effect on TLR4 and TLR9 signaling. Indeed, LPS or CpG stimulation did not up-regulate IL-12 or T cell stimulatory activity of liver pDC from MDP-exposed animals to the same extent as that of control mice. These data are consistent with higher expression of NOF2 and IRAF in liver pDC, which suggests an inhibitory effect of IRF-4 on the signaling cascade leading to NF-κB activation. Notably, liver mDC that expressed lower levels of NOD2 compared with pDC did not up-regulate IRF-4 after MDP treatment, and TLR4-induced IL-12 production and T cell stimulatory activity after LPS stimulation were similar to that of control mDC, although the inherent ability of liver mDC to up-regulate IL-12 secretion after maturation in culture was impaired. This suggests that signaling events in liver mDC downstream of NOD2 and TLR ligation may differ from those in liver pDC.

MDP treatment also impaired the CpG-induced T cell stimulatory ability of liver pDC. The analysis of splenic DC subsets we conducted did not show any significant changes, which is consistent with an important influence of the liver microenvironment on DC responsiveness. Indeed, we found that unlike in vivo, MDP had little direct effect on liver pDC function in vitro. It has been reported for pDC that engagement of TLR9 by CpG, leading to IFN-α secretion, involves a multiprotein signal-transducing complex that includes MyD88, TRAF6, and IRF-7 (67). MyD88 and TRAF6 are inhibited by IRF-4 (31), which we found was increased in liver but not spleen pDC after systemic MDP treatment. Consistent with the expression of IRF-4, the stimulation of liver pDC from MDP-treated animals with CpG did not induce the same level of IFN-α secretion as that from control pDC. By contrast, expression of IRF-4 in spleen pDC from MDP-treated mice did not differ from that of the control group, and IFN-α secretion after CpG stimulation was higher (data not shown). Consistent with these ex...
by stimulating hepatic interleukin-6/signal transducer and activator of transcription 3 activity. 


