Low TLR4 Expression by Liver Dendritic Cells Correlates with Reduced Capacity to Activate Allogeneic T Cells in Response to Endotoxin

An De Creus, Masanori Abe, Audrey H. Lau, Holger Hackstein, Giorgio Raimondi and Angus W. Thomson

*J Immunol* 2005; 174:2037-2045; doi: 10.4049/jimmunol.174.4.2037
http://www.jimmunol.org/content/174/4/2037

References
This article cites 57 articles, 27 of which you can access for free at: http://www.jimmunol.org/content/174/4/2037.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Low TLR4 Expression by Liver Dendritic Cells Correlates with Reduced Capacity to Activate Allogeneic T Cells in Response to Endotoxin

An De Creus,* Masanori Abe,* Audrey H. Lau,* Holger Hackstein,† Giorgio Raimondi,* and Angus W. Thomson2*†

Signaling via TLRs results in dendritic cell (DC) activation/maturation and plays a critical role in the outcome of primary immune responses. So far, no data exist concerning TLR expression by liver DC, generally regarded as less immunostimulatory than secondary lymphoid tissue DC. Because the liver lies directly downstream from the gut, it is constantly exposed to bacterial LPS, a TLR4 ligand. We examined TLR4 expression by freshly isolated, flow-sorted C57BL/10 mouse liver DC compared with spleen DC. Real-time PCR revealed that liver CD11c+CD8α− (myeloid) and CD11c+CD8α+ (“lymphoid-related”) DC expressed lower TLR4 mRNA compared with their splenic counterparts. Lower TLR4 expression correlated with reduced capacity of LPS (10 ng/ml) but not anti-CD40-stimulated liver DC to induce naïve allogeneic (C3H/HeJ) T cell proliferation. By contrast to LPS-stimulated splenic DC, these LPS-activated hepatic DC induced alloantigen-specific T cell hyporesponsiveness in vitro, correlated with deficient Th1 (IFN-γ) and Th2 (IL-4) responses. When higher LPS concentrations (>100 ng/ml) were tested, the capacity of liver DC to induce proliferation of T cells and Th1-type responses was enhanced, but remained inferior to that of splenic DC. Hepatic DC activated by LPS in vivo were inferior alloantigen T cell stimulators compared with splenic DC, whereas adoptive transfer of LPS-stimulated (10 ng/ml) liver DC induced skewing toward Th2 responses. These data suggest that comparatively low expression of TLR4 by liver DC may limit their response to specific ligands, resulting in reduced or altered activation of hepatic adaptive immune responses. The Journal of Immunology, 2005, 174: 2037–2045.

Dendritic cells (DC) are uniquely well-equipped, bone marrow-derived APC that link innate and adaptive immunity. Immature DC in peripheral tissues take up and process Ags, undergo maturation, and migrate to T cell areas of secondary lymphoid organs. Therein they activate naïve T cells and initiate immune reactivity (1). Multiple DC subsets have been identified in mice and humans (2), in particular murine classical CD11c+CD8α− (myeloid), CD11c+CD8α+ (“lymphoid-related”), and plasmacytoid DC (CD11c−/B220+CD19−), which are found both in lymphoid and nonlymphoid tissues, including the liver (3–7).

Recognition by DC of pathogen-associated molecular patterns or pathogen-derived products, such as LPS from Gram-negative bacteria, lipoteichoic acid or bacterial CpG-DNA, involves specific receptors, in particular TLR, highly conserved type-1 transmembrane proteins identified initially in Drosophila (8–11). Signaling via TLR induces distinct patterns of gene expression that not only lead to the activation of innate immunity, but also instruct the development of Ag-specific acquired immunity (11). TLR4 was first implicated in the recognition of LPS (12), whereas more recent studies have shown that structurally distinct LPS from certain bacteria can signal through TLR2 (13). Recognition of LPS is determined by interaction between multiple components, i.e., LPS-binding protein, CD14 and the TLR4-myeloid differentiation protein-2 (MD-2) complex (14). Signaling via TLR induces DC activation and the secretion of numerous cytokines (15–17), initiating and shaping the adaptive immune response (18). There is also evidence that microbial products (LPS, CpG) that engage TLR on mouse DC can block the suppressive effect of CD4+CD25+ T regulatory cells, allowing efficient activation of pathogen-specific immune responses (19).

Distinct human DC subsets express different repertoires of TLR and thus respond to different pathogen-associated molecular patterns from a variety of pathogens. For example, myeloid and plasmacytoid DC express TLR4 and TLR9, respectively (20–22). In mice, LPS and CpG (TLR9 ligand) differentially affect the ability of distinct DC subsets to direct Th cell responses, as a result of their differential expression of TLR4 and TLR9. At high Ag doses, Th1 development is not enhanced by LPS- or CpG-stimulated DC, whereas Th1 responses are promoted by these TLR ligands in the presence of low Ag doses (23). These data suggest that DC subsets, Ag dose, stage of DC maturation, and microenvironmental signals determine the outcome of immune responses. In addition to a role for TLR in determining the balance between Th1 and Th2 responses, a role for TLR4 in mediating innate IL-10 secretion to activate T regulatory cells has been suggested (24).

Immune responses in the liver preferentially result in tolerance rather than immunity (25–27) and liver DC progenitors exhibit...
tolerogenic properties (28, 29). So far, no data exist on TLR expression by liver DC. We hypothesized that the tolerogenic environment of the liver, that is rich in IL-10 and TGF-β (30, 31), might affect the expression of TLRs by intrahepatic DC, thereby altering their response to specific ligands, in particular LPS. Because the liver is located downstream from the gut, it is constantly exposed to LPS. Our findings reveal that liver CD8α+ and CD8α− DC express lower levels of TLR4 mRNA than their splenic counterparts. These discrepancies between liver and spleen DC could, however, be at least partially overcome by exposure to higher concentrations of LPS that exceed those encountered (in humans) in the absence of infection (32).

Materials and Methods

Animals

Ten- to 12-wk-old C57BL/J10 (B10; H2Kb), BALB/c (H2Kd), and C3H/HeJ (C3H; H2Kd) male mice were purchased from The Jackson Laboratory. They were maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh Medical Center. They received Purina rodent chow (Ralston Purina) and tap water ad libitum. Experiments were conducted in accordance with the National Institutes of Health guide for use and care of laboratory animals and under an Institutional Animal Care and Use Committee-approved protocol.

Media, reagents, and Abs

RPMI 1640 medium was supplemented with 10% v/v heat-inactivated FCS (Nalgene), 0.1 mM nonessential amino acids, 2 mM t-glutamine, sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (complete medium) (all reagents from Invitrogen Life Technologies). Chinese hamster ovary cell-derived recombinant fms-like tyrosine kinase 3 ligand (Flt3L) was provided by Amgen. Purified LPS (Escherichia coli O55:B5) was obtained from Sigma-Aldrich. MAbS used for flow cytometry and cell sorting were hamster anti-mouse CD11c (HL3; biotin- or PE-conjugated), rat anti-CD8α (53-6.7; biotin-conjugated), anti-CD11b (M1/70; FITC-conjugated), anti-CD19 (1D3; FITC-conjugated), anti-B220 (RA3-6B2; CyChrome-conjugated), anti-programmed death ligand 1 (PD-L1) (MH5; PE-conjugated; eBioscience), anti-CD11c (98.125; PE-conjugated; eBioscience), anti-rat mouse CD80 (16-10A1; PE-conjugated), anti-rat anti-mouse CD86 (GL1; PE-conjugated) and mouse anti-IAβ-β chain (APF-120.1; PE-conjugated) (all mAbs from BD Pharmingen, unless specified). Low endotoxin hamster IgM anti-CD40 mAb (HM40-3) and control matched Ig (BD Pharmingen) served as negative controls. After washing, biotin-conjugated mAbs were revealed with second step streptavidin-CyChrome (BD Pharmingen). The cells were then analyzed using a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter). Liver and spleen Ny-codenz-enriched DC suspensions were incubated with anti-CD11c-PE, anti-CD86 (GL1; PE-conjugated), anti-CD19-FITC, anti-CD11b FTTC, and anti-B220 CyChrome mAbs at 4°C for 45 min. CD11c+CD8α−, CD11c+CD8α+, and CD11c+lowB220+CD11b+ CD19− were then sorted to >99% purity using a Coulter EPICS Elite cell sorter (Beckman Coulter).

Real-time RT-PCR for TLR4

Real-time RT-PCR was performed on an ABI Prism 7000 PCR cycler (Applied Biosystems) as described (33). The following validated PCR primers and TaqMan MGB probes (FAM labeled) were used: murine TLR4 (Assay ID: Mm00445274_m1) and 18S ribosomal RNA (Assay ID: Hs99999901_s1) as endogenous control. PCR mix was prepared according to the manufacturer’s instructions (Assay on demand; Applied Biosystems) and thermalycler conditions were as follows: 1 × 2 min 50°C, 1 × 10 min 95°C, 40–50 cycles denaturation (15 s, 95°C) and combined annealing/extension (1 min, 60°C). Relative expression of the TLR4 gene was calculated using the ddct method (2^ddCT) after normalization to the endogenous control 18S RNA and calibration to CD8α+ spleen DC as reference.

Mixed lymphocyte reaction

Spleen cell suspensions from C3H mice were depleted of RBC by NH4Cl treatment and resuspended in warm (37°C) complete medium. The cell suspension was passed over a nylon wool column to enrich for T cells (purity >85%). A total of 2 × 10^6 nylon wool column-purified C3H T cells were stimulated with graded numbers of irradiated (20 Gy) control or LPS-stimulated B10 liver or spleen CD11c+ DC or with sorted, control or LPS-stimulated liver or spleen CD8α+ or CD8α− DC in complete medium in round-bottom, 96-well plates (Corning). [3H]Thdr (1 μCi) was added to each well for the final 16 h of 72-h cultures. Cultures were harvested using a multiple well harvester and [3H]Thdr uptake determined using a liquid scintillation counter. Tests were conducted in triplicate, and results expressed as mean cpm ± 1 SD.

Assessment of T cell hyporesponsiveness

A total of 2 × 10^6 C57BL/10 T cells were first cocultured with 2 × 10^5 LPS-stimulated B10 liver or spleen bulk CD11c+ DC. Five days later, the T cells were recovered and rested for 72 h in complete medium containing 5 U/ml rIL-2. Subsequently, the T cells (2 × 10^5) were restimulated in the presence or absence of 25 U/ml rIL-2, with splenocytes (2 × 10^5) from the same donor strain (B10) as in the primary culture, or from a third party donor (BALB/c). Proliferation was measured 72 h later by [3H]Thdr incorporation. Tests were conducted in triplicate, and results expressed as mean cpm ± 1 SD. Cytokine levels in supernatants of restimulated T cell cultures were measured by ELISA 24 or 72 h after the start of the cultures.

In vivo LPS administration

Different doses of LPS (10 ng or 1 μg/mouse in 0.2 ml of PBS) were injected via the lateral tail vein of B10 mice. Twelve hours later, liver and spleen DC were isolated and used as stimulators in primary MLR.

Adoptive DC transfer

A total of 3 × 10^6 purified LPS-stimulated B10 liver or spleen bulk CD11c+ DC were injected s.c. into the hind footpads of normal allogeneic C3H mice. Five days later, popliteal lymph node cell suspensions were prepared and cells cultured in 96-well, round-bottom plates (Corning) at 5 × 10^5 cells/well in complete medium in the presence of 5 × 10^5 B10 or BALB/c splenocytes. After 24 or 72 h, supernatants were harvested and cytokine concentrations measured by ELISA. T cell proliferation was measured by [3H]Thdr incorporation during the last 16 h of the 3-day cultures.

Cytokine quantitation

IFN-γ, IL-10, and IL-4 were quantified by ELISA using commercial kits from R&D Systems and following the manufacturer’s recommended procedures. The detection limit was 2.0 pg/ml.

Flow cytometry and cell sorting

Flow cytometric analyses and sorting were performed as described (3) with minor modifications. To avoid nonspecific Ab binding, cells were preincubated with 10% v/v normal goat serum for 20 min at 4°C. Control or LPS-stimulated, CD11c-enriched cells were incubated with the indicated mAbs for 45 min at 4°C. Cells incubated with the appropriate isotype-matched Ig (BD Pharmingen) served as negative controls. After washing, biotin-conjugated mAbs were revealed with second step streptavidin-CyChrome (BD Pharmingen). The cells were then analyzed using a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter). Liver and spleen Ny-codenz-enriched DC suspensions were incubated with anti-CD11c-PE, anti-CD86 (GL1; PE-conjugated), anti-CD19-FITC, anti-CD11b FTTC, and anti-B220 CyChrome mAbs at 4°C for 45 min. CD11c+CD8α−, CD11c+CD8α+, and CD11c+lowB220+CD11b+ CD19− were then sorted to >99% purity using a Coulter EPICS Elite cell sorter (Beckman Coulter).
Statistics

The significance of differences between means was determined using Student’s unpaired t test or Fisher’s protected least significant difference test. A value of \( p < 0.05 \) was considered significant.

Results

Splenic but not liver DC exposed to low LPS concentrations strongly induce allogeneic T cell proliferation in vitro

To obtain DC, mice were pretreated with the endogenous DC poietin Flt3L, as described in Materials and Methods. DC mobilization facilitated their recovery, greatly enhanced their yield and minimized the numbers of animals used. To determine whether freshly isolated liver DC compared with splenic DC would respond differently to LPS in vitro, immunobead-purified B10 liver or spleen bulk CD11c⁺ DC were cultured in low concentration LPS (10 ng/ml). After overnight (18 h) culture, the DC were harvested and tested for their capability to stimulate naive allogeneic (C3H) T cells. LPS-activated splenic CD11c⁺ DC were very efficient in stimulating T cells, whereas only weak T cell proliferation was induced by LPS-activated liver DC (Fig. 1A). Similar results were obtained for flow-sorted liver CD11c⁺CD8α⁺ and CD11c⁺CD8α⁻ DC subsets compared with their splenic counterparts as for bulk liver CD11c⁺ DC (Fig. 1B).

Differential expression of TLR4 on mouse liver and spleen DC subsets

The comparatively poor response of liver DC to low concentration LPS (10 ng/ml) prompted us to examine the expression of TLR4 by these APC because it has been shown that LPS acts through TLR4 to induce DC maturation (12, 34). Corresponding splenic DC were used as reference. Results are from one experiment representative of two performed using independent sets of flow-sorted cells. As a control, we compared the expression of TLR4 between splenic and liver CD8⁺ CD11c⁺ DC (that showed differences in DC subset composition between the tissues (4)) to determine whether the differential response of splenic vs liver DC to LPS was due to a difference in TLR4 expression, and to ascertain which liver DC subset(s) was most likely to contribute to the deficient capacity of bulk liver DC to stimulate allogeneic T cells, we flow-sorted liver and splenic CD11c⁺CD8α⁺, CD11c⁺CD8α⁻, and CD11clowB220⁺CD11b⁺ CD19⁺ DC from Flt3L-treated mice (purity >99%) and examined the expression of TLR4 mRNA by quantitative real-time PCR. Splenic CD8α⁺ and CD8α⁻ DC expressed higher (~3- to 5-fold) TLR4 mRNA levels compared with liver CD8α⁻ and CD8α⁻ cells (Fig. 2). By contrast, splenic and liver CD11clowB220⁺ DC showed similar mRNA expression levels.

Higher concentrations of LPS overcome deficient allogeneic T cell proliferation induced by liver DC, correlating with a more mature DC phenotype

To examine whether higher concentrations of LPS could enhance the capacity of liver DC to stimulate allogeneic T cells, bead-purified liver B10 CD11c⁺ DC were cultured in increasing concentrations of LPS (10 ng-1 μg/ml). Because plasmacytoid DC from both liver and spleen expressed low but similar amounts of mRNA for TLR4 (Fig. 2), we only examined the response of liver and spleen CD8α⁺ and CD8α⁻ DC (that showed differences in TLR4 expression) to these different concentrations of LPS. After overnight culture, CD11c⁺ DC were harvested and tested for their ability to stimulate allogeneic T cells. B10 spleen CD11c⁺ DC were used as controls. As shown in Fig. 3A, the capacity of B10 liver CD11c⁺ DC to stimulate naïve allogeneic T cells was enhanced significantly when higher concentrations of LPS were used. Notably, however, their stimulatory capacity at comparatively high concentrations was still significantly lower than that of spleen DC.
stimulator to responder ratios remained inferior to that of splenic DC (Fig. 3A). Similar results were obtained for flow-sorted liver CD8α− and CD8α+ DC as for bulk liver CD11c+ DC (Fig. 3B).

To determine whether liver CD11c+ were refractory to LPS-induced maturation, we analyzed the surface phenotype of liver and splenic CD11c+ DC stimulated overnight (18 h) with graded doses of LPS by flow cytometric analysis. Mature DC are known to express high levels of MHC class II and B7 family costimulatory molecules (CD80 and CD86) compared with immature DC. Expression of these molecules was analyzed by gating on CD11c+ cells. It is evident in Fig. 3C that liver DC stimulated overnight with 10 ng/ml LPS expressed lower levels of MHC class II (IAb), CD80, and CD86 compared with splenic DC. A higher dose of LPS (1 μg/ml) increased the intensity of expression of MHC class II, CD80, and CD86 on liver DC, but these values were also below those of similarly stimulated spleen DC. Programmed death ligand 1 and 2 (PD-L1/2 = B7-H1/DC) expression, that has been implicated in regulation of T cell responses (35, 36) and the deletion of intrahepatic T cells (37), was similar on liver and splenic CD11c+ cells, irrespective of the concentration of LPS. These observations on B7 family cosignaling molecule expression are in agreement with the increased ability of liver DC to activate allogeneic T cells when higher stimulatory doses of LPS are used (Fig. 3B).

Liver and splenic DC stimulated by CD40 ligation exhibit similar T cell allostimulatory activity

To ascertain whether differences in the allostimulatory function of liver and splenic DC might also be observed following their activation independent of TLR4 ligation, we exposed B10 bulk CD11c+ immunobead-sorted liver or spleen DC to various concentrations of agonistic anti-CD40 mAb (10 ng-1 μg/ml) for 24 h.
before using the cells as stimulators of naive C3H T cells in primary MLR. As shown in Fig. 4, and in contrast to LPS activation, no significant difference was observed between the T cell proliferative responses induced by anti-CD40-stimulated liver and spleen DC.

**Allogeneic T cells stimulated by LPS-activated splenic or liver DC, show differential ability to secrete IFN-γ and IL-4**

Recently, it has been shown that high levels of TLR4 mRNA expression by DC can, in the presence of Ag, skew Th cell responses toward Th1 upon LPS stimulation (23). To determine whether, in the presence of alloantigen, the lower expression of TLR4 by liver DC could, depending on the level of LPS, determine the outcome of a T cell response, we analyzed the secretion of IFN-γ and IL-4 by allogeneic T cells after restimulation with Ag. Naïve allogeneic C3H T cells were cultured with LPS-activated B10 liver or spleen CD11c+ DC. After 5 days, the T cells were recovered and cultured for an additional 3 days in the presence of rIL-2 (5 U/ml). Twenty-four or 72 h after restimulation with freshly isolated B10 splenocytes, the culture supernatants were harvested and Th1/Th2 cytokines measured. Liver DC activated initially in the presence of low concentration LPS (10 ng/ml) induced much lower levels of IFN-γ and IL-4 production by allogeneic T cells after their restimulation compared with spleen DC (Fig. 5). The levels of IFN-γ produced by liver DC-stimulated T cells increased significantly with higher concentrations of LPS (>100 ng/ml), whereas the levels induced by spleen DC remained very similar. By contrast, IL-4 secretion induced in allogeneic T cells by spleen DC was reduced significantly with higher LPS concentrations, indicating a more pronounced Th1-type response. This Th1-type skewing response (IFN-γ > IL-4) was induced in liver DC-stimulated T cells only at comparatively high LPS concentrations. Thus the threshold level of LPS needed to condition liver DC to induce an allogeneic Th1-type response was higher than for spleen DC (Fig. 5).

**Induction of alloantigen-specific T cell hyporesponsiveness by LPS-activated liver DC**

To determine whether low concentration LPS-activated (10 ng/ml) liver DC could induce alloantigen-specific T cell hyporesponsiveness, we used a two-step assay, as described in Materials and Methods. First, purified allogeneic C3H T cells were cultured with LPS-activated B10 liver or control spleen CD11c+ DC. After 5 days, the T cells were recovered, maintained for an additional 3 days in the presence of rIL-2 (5 U/ml), then restimulated with fresh B10 splenocytes, with or without exogenous rIL-2 (25 U/ml). Allogeneic T cells cultured with LPS-activated spleen DC showed strong proliferative responses upon restimulation with B10 splenocytes during the second culture, whereas those cultured with LPS-activated liver DC showed less vigorous proliferation following restimulation. Addition of IL-2 at the start of the second MLR partially reversed this hyporesponsiveness (Fig. 6).

To ascertain whether the induction of T cell hyporesponsiveness was Ag-specific, fresh splenocytes from third party (BALB/c) mice were used for restimulation. No difference in allogeneic T
cell proliferation was observed, independent of the origin of the DC (Fig. 6), indicating that the unresponsiveness induced by low concentration LPS-activated liver DC was Ag-specific.

**Systemic administration of different doses of LPS matures splenic but not liver DC**

To determine the capacity of liver DC to mature in vivo in response to LPS, different doses of LPS (10 ng or 1 μg) were injected via the lateral tail vein of normal B10 mice. Twelve hours later, bulk CD11c+ liver or splenic DC were harvested, purified, and tested for their ability to stimulate naive allogeneic (C3H) T cells. The capacity of low dose in vivo LPS-activated liver CD11c+ DC to stimulate allogeneic T cells was significantly lower than that of spleen CD11c+ DC (Fig. 7). Interestingly, a higher dose of systemic LPS did not override the inferior ability of liver DC to stimulate allogeneic T cells ex vivo.

**Adoptively transferred, LPS-stimulated liver DC can induce skewing toward Th2-type responses**

To determine whether low-dose LPS-stimulated liver DC were also impaired in inducing Th1-type responses in vivo, B10 bulk liver CD11c+ DC (or control spleen DC) were stimulated overnight with 10 ng/ml LPS then injected s.c. into the hind footpads of naive allogeneic (C3H) mice. Five days later, draining lymph node cells were isolated and restimulated in vitro with fresh B10 splenocytes. As shown in Fig. 8, adoptive transfer of LPS-stimulated liver or splenic DC resulted in differential priming of draining lymph node T cells. Following their s.c. injection, the LPS-stimulated liver DC were less efficient in priming allogeneic T cells than similarly stimulated splenic DC, as demonstrated by the ex vivo proliferative responses of the T cells to restimulation with donor alloantigens (Fig. 8A). Next, Th1/Th2 cytokines released in the culture supernatants were measured. Previously, we showed that adoptive transfer of unstimulated liver DC precursors resulted in the activation of allogeneic T cells that preferentially secreted IL-4 and less IFN-γ (29). As shown in Fig. 8, B–D, liver DC stimulated with 10 ng/ml LPS primed allogeneic T cells to secrete significantly lower levels of IFN-γ (Fig. 8B) and higher levels of IL-10 and IL-4 (Fig. 8, C and D) than those primed by similarly stimulated splenic DC. This difference was lost when a 100-fold higher concentration of LPS (1 μg/ml) was used to activate the DC. Thus at comparatively low concentrations, LPS induces liver DC to skew toward Th2-type responses.

**Discussion**

The refractoriness of the liver in terms of its role in immune stimulation may have evolved in concert with the need for immunologic unresponsiveness (tolerance) to orally acquired Ag and microbial products derived from the intestine in the normal steady state. The relative T cell unresponsiveness, that may reflect comparative functional deficiencies in DC or other hepatic APC, such as sinusoidal endothelial cells (38), may at least in part, explain the persistence of some hepatic viral infections, cancer metastasis to the liver, the tolerogenicity of liver allografts (39, 40), oral tolerance and the tolerogenicity of portal venous-infused Ag (41, 42). Although the immunologic mechanism(s) underlying liver tolerogenicity remain poorly defined, attention has recently focused on hepatic APC (4, 31, 38, 43) and intrahepatic T cell fate and function (26, 44, 45).

In this study, we have demonstrated that, consistent with comparatively low expression of TLR4, LPS-stimulated murine liver DC are inferior stimulators of naive, allogeneic T cells compared with splenic DC, both in vitro and in vivo. Liver DC stimulated with comparatively low concentrations of LPS (10 ng/ml) are nototypically immature, as determined by surface MHC and B7 family costimulatory molecule expression, and can induce alloantigen-specific T cell hyporesponsiveness. They also exhibit inferior capacity, compared with similarly stimulated splenic DC, to induce alloimmune Th1 responses in vitro and in vivo and can skew toward Th2-type responses.

Our results extend earlier observations (4, 29, 43, 46) that liver DC or their progenitors differ from bone marrow-derived and secondary lymphoid tissue DC, the latter typified by those in the spleen. Thus, liver DC (bulk DC and CD8α− and CD8α+ subsets) expressed lower levels of TLR4 compared with similarly isolated and purified splenic DC that correlated with their inferior responses to LPS. Conceivably, within the hepatic microenvironment, constitutive production of anti-inflammatory mediators, such as IL-10 and TGF-β, by various cell types, may alter the T cell stimulatory potential of liver DC (31) and other intrahepatic APC by modulating their differentiation, maturation and function. Others (47) have demonstrated that TLR4 message is increased in TGF-β null mice, in association with hyperresponsiveness to LPS, suggesting a regulatory link between TGF-β and TLR4 expression. Because the liver microenvironment is exceptionally rich in TGF-β compared with the spleen (30, 45), the lower expression of TLR4 mRNA by liver DC compared with spleen DC could result from constitutive high intrahepatic concentrations of TGF-β. In addition, there is evidence that persistent signaling through TLR is necessary to bypass regulatory T cell-mediated tolerance (48). These findings suggest that continuous stimulation of DC via TLR4 is necessary for sustained proinflammatory cytokine production required for reversing regulatory T cell-mediated tolerance. Therefore, like intestinal epithelium, which exhibits low or absent TLR4 expression (49), low expression of TLR4 mRNA by liver DC compared with spleen DC could result from constitutive high intrahepatic concentrations of TGF-β. In addition, there is evidence that persistent signaling through TLR is necessary to bypass regulatory T cell-mediated tolerance (48). These findings suggest that continuous stimulation of DC via TLR4 is necessary for sustained proinflammatory cytokine production required for reversing regulatory T cell-mediated tolerance. Therefore, like intestinal epithelium, which exhibits low or absent TLR4 expression (49), low expression of TLR4 mRNA by liver DC compared with spleen DC could result from constitutive high intrahepatic concentrations of TGF-β. In addition, there is evidence that persistent signaling through TLR is necessary to bypass regulatory T cell-mediated tolerance (48). These findings suggest that continuous stimulation of DC via TLR4 is necessary for sustained proinflammatory cytokine production required for reversing regulatory T cell-mediated tolerance. Therefore, like intestinal epithelium, which exhibits low or absent TLR4 expression (49), low expression of TLR4 mRNA by liver DC compared with spleen DC could result from constitutive high intrahepatic concentrations of TGF-β. In addition, there is evidence that persistent signaling through TLR is necessary to bypass regulatory T cell-mediated tolerance (48). These findings suggest that continuous stimulation of DC via TLR4 is necessary for sustained proinflammatory cytokine production required for reversing regulatory T cell-mediated tolerance. Therefore, like intestinal epithelium, which exhibits low or absent TLR4 expression (49), low expression of TLR4 mRNA by liver DC compared with spleen DC could result from constitutive high intrahepatic concentrations of TGF-β. In addition, there is evidence that persistent signaling through TLR is necessary to bypass regulatory T cell-mediated tolerance (48). These findings suggest that continuous stimulation of DC via TLR4 is necessary for sustained proinflammatory cytokine production required for reversing regulatory T cell-mediated tolerance. Therefore, like intestinal epithelium, which exhibits low or absent TLR4 expression (49), low expression of TLR4 mRNA by liver DC compared with spleen DC could result from constitutive high intrahepatic concentrations of TGF-β.
the anti-inflammatory microenvironment within the liver. Active down-regulation of TLR4 expression might result from constant exposure to anti-inflammatory cytokines (TGF-β, IL-10) and/or to comparatively high levels of LPS in the liver, as a mechanism to prevent constant liver DC activation. Our data show that the relative unresponsiveness of liver DC to LPS can be partially overcome when higher concentrations of LPS are used, indicating that TLR4 expression is functional on liver DC, and that these important hepatic APC can respond to TLR ligands when, in the case of LPS, concentrations are elevated significantly above those encountered (in humans) in the absence of clinical infection (32).

We have demonstrated previously (29) that unstimulated, murine liver-derived DC progenitors preferentially induce IL-10- and IL-4-secreting T cells (Th2), both in vitro and in vivo, whereas DC propagated from bone marrow preferentially induce IFN-γ-secreting T cells (Th1). These data, together with those concerning low dose LPS-stimulated liver DC we have reported, suggest that poor induction of Th1 responses and skewing toward Th2-type responses by hepatic DC might contribute to the inherent tolerogenicity of the liver. Others have shown that the relative levels of inhibitory PD-L1 and costimulatory B7-1/B7-2 signals on APC may determine the extent of T cell activation and consequently, the threshold between tolerance and autoimmunity (36). We have shown in this study that liver DC stimulated with low concentrations of LPS express similar levels of PD-L1/2, but lower levels of CD80 and CD86 compared with spleen DC stimulated with similar concentrations of LPS. Based on these data, we speculate that the expression of PD-L1/2 on liver DC might also contribute to their tolerogenic properties by preventing T cell activation. In addition, it has been shown that expression of PD-L1 may play a role in the induction of T cell anergy (53). Our data indeed reveal that liver DC stimulated with low doses of LPS induce T cell hyporesponsiveness, thus a comparatively high level of PD-L1 relative to CD80/CD86 on these cells may promote the induction of hyporesponsiveness/anergy. The data we obtained for anti-CD40-dependent maturation of spleen and liver DC suggest that signals derived from T cells should be sufficient to fully mature liver DC. However, it must be borne in mind that the concentrations of anti-CD40 we used to mature DC cannot be compared with those of LPS. Liver DC stimulated with low levels of LPS cannot be further matured in the presence of allogeneic T cells during culture, suggesting that signals derived from T cells are not able to compensate for the inherent low stimulatory capacity of these APC. This suggests that the anti-CD40 concentrations used to mature liver DC are probably too high to visualize differences between these and spleen DC.

The liver is considered the least immunogenic of transplanted whole organs and in mice, can induce robust, donor-specific tolerance, in the absence of anti-rejection therapy (26, 40). Following liver transplantation, sustained release from the graft of immature DC, refractory to TLR4 ligation and with poor comparative ability to stimulate Th1 responses, may contribute to tolerance induction. Migration of these DC to secondary lymphoid tissue (46) may promote the generation of Th2 and/or regulatory T cell clones with capacity to down-regulate Th1 responses and maintain the state of donor-specific unresponsiveness.

Once ligated, TLRs initiate a signaling pathway via their universal adaptor protein, Myd88 (54). Activation of this pathway induces NF-κB translocation, which in DCs, initiates up-regulation of costimulatory molecules and the release of proinflammatory cytokines (55). This pathway is critical for Th1 immunity (54). Recently, evidence has been presented that Myd88 can be activated in the setting of organ transplantation (56) by as yet undefined ligands, and not solely by infections. Moreover, it appears that the

FIGURE 8. Adoptively transferred, low concentration-stimulated liver DC induce an inferior Th1 response compared with splenic DC and skew toward Th2-type responses. Bulk B10 CD11c+ DC were stimulated overnight with 10 ng/ml or 1 μg/ml LPS, then injected s.c. into naïve C3H recipients. Five days later, anti-donor proliferative responses (A) of draining lymph node T cells and both Th1 (IFN-γ) (B) and Th2 cytokine (IL-10, IL-4) production (C and D) were determined, as described in Materials and Methods. Results are means ± 1 SD obtained from one experiment representative of three performed. *, p < 0.01; **, p < 0.05.
alloimmune response may be critically dependent on signaling via MyD88. However, TLR4-/- mice do not exhibit a delay in (skin) allotraft rejection (56). Conversely, it has been shown that LPS signaling is activated during ischemia reperfusion injury in a rotenone model of liver transplantation (57).

Different types of DC express distinct sets of TLRs. In the present study, we found that both classical myeloid (CD14+/CD16+) and “lymphoid-related” liver DC (CD16+/CD16-) expressed low levels of TLR4, whereas freshly isolated liver plasmacytoid DC (B220+/CD123+) expressed comparatively high levels of TLR9 (data not shown). Recently, Lian et al. (6) have reported that only small amounts of CpG oligonucleotide sequences with high induction of IFN-α/β in plasmacytoid dendritic cells. Eur. J. Immunol. 31:2154.


