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Distinct Response of Liver Myeloid Dendritic Cells to Endotoxin is Mediated by IL-27

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Abstract

Background—The liver lies downstream of the gut, and is constantly exposed to bacteria. Liver dendritic cells (DC) are known to possess properties of tolerance, and respond to LPS differently when compared to conventional DC, but the underlying mechanisms are not completely understood.

Methods—To enhance yield number, liver DC were isolated from the mice that had rapidly been injected with plasmid-GM-CSF through tail vein, and characterized the expression of TLR4 in response to LPS stimulation (qPCR), cytokine production (ELISA and qPCR) and ability to elicit T cell response (MLR). The results were compared to spleen DC.

Results—The threshold of LPS stimulation for liver DC was markedly higher than spleen DC, even though the expression of TLR4 on both DCs was comparable. In contrast to spleen DC that produced high levels of IL-12 and induced Th1 response upon LPS stimulation, LPS-liver DC preferentially produced IL-10 and IL-27, instead of IL-12. In addition, liver DC induced T cell hyporesponsiveness, associated with selective expansion of CD4⁺Foxp3⁺ T regulatory cells. Addition of exogenous IL-12 only slightly enhanced liver DC-induced T cell response. Interestingly, abrogation of IL-27 ligation by using IL-27R^{-/-} T cells synergistically augmented the effect of IL-12, suggesting that IL-27 produced by liver DC plays a crucial role in induction of T cell hyporesponsiveness.

Conclusions—Liver DC respond distinctly to LPS stimulation by secreting IL-27 which synergizes with silencing of bioactive IL-12 activity leading to profound T cell inhibition.

Keywords

TLR4; T cell hyporesponsiveness; Regulatory T cells

Introduction

Hepatic tolerance was initially demonstrated by spontaneous acceptance of liver transplants in many species without requirement of immunosuppressive therapy (1–3). In clinical practice, liver transplant recipients require less immunosuppression than recipients of other solid organ allografts (4). Many rejection episodes in human liver transplants are self-limiting and do not require additional high-dose immunosuppression (5,6). Also, weaning of immunosuppression

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is feasible in case of liver allograft recipients, but is a difficult proposition for other organ transplants (7). In addition, infusion of antigens into the portal vein to induce immune tolerance and the requirement of antigen transport to the liver for induction of oral tolerance (8) suggest existence of immune modulation within the liver.

DC residing in a tolerizing milieu, such as in the liver, Peyer's patches of the small intestine, or the anterior chamber of eyes, secrete IL-10 but not IL-12 (9–11). Although the precise mechanism is not clear, these cells can generate "tolerogenic" activity upon processing antigens by inducing T cell anergy (12) and facilitating expansion of CD4⁺CD25⁺ Treg cells (8). The liver lies directly downstream from the gut, and is constantly exposed to harmless food antigens as well as components of commensal gut bacteria, such as lipopolysaccharide (LPS) (13,14). In this study, we have demonstrated a distinct response pattern of liver DC to stimulation by LPS, a ligand for TLR4. In contrast to spleen DC that produced high levels of IL-12 and induced enhanced Th1 response in allogeneic T cells; liver DC, in spite of comparable TLR4 expression, preferentially secreted IL-10 and IL-27 instead of IL-12. Although LPS-stimulated liver DC were phenotypically mature, they induced T cell hyporesponsiveness. This effect was not simply due to lack of IL-12 since addition of exogenous IL-12 did not significantly reverse liver DC induced T cell hyporesponsiveness. We did however note that abrogation of the IL-27 pathway largely restored the T cell responses to IL-12, suggesting a crucial immunomodulatory role of IL-27 produced by liver DC.

Materials and Methods

Animal

8–12 week-old male C57BL/6 (B6; H-2^b), BALB/c (H-2^d) and C3H (H-2^k) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-27 receptor (R) WSX-1 knockout mice were provided by Amgen (Seattle, WA). All animals were maintained in the specific pathogen-free facility at Lerner Research Institute, Cleveland Clinic, and used in accordance with institutional and NIH guidelines.

Isolation of liver DC

To enhance the yield number of liver DC, we transfected a plasmid containing the GM-CSF gene driven by a CMV promoter into mouse livers by tail vein rapid injection as previously described (15). Briefly, 20 µg plasmid-GM-CSF in 2.0 ml PBS was tail vein injected at a rate of 0.3 ml/sec. Seven days later, liver nonparenchymal cells (NPC) were isolated, and mononucleocyte enriched population was separated by Percoll (Sigma-Aldrich, Milwaukee, WI) gradient centrifugation. DC were purified by CD11c positive sorting using magnetic beads with a purity of 93–96% determined by flow analysis. Spleen DC from the same mouse were similarly isolated for comparison. For stimulation, DC were exposed to LPS at 0.1 or 2.5 µg/ml (L-2880, Sigma-Aldrich) for 18 hours.

mAbs and Flow cytometry

The mAbs against CD3, CD11b (rat IgG_{2b}), CD4 and CD86 (both rat IgG_{2a}), CD25 (rat IgG1), CD11c, CD40, CD54, CD80 (all hamster IgG), H-2K^b (mouse IgG_{2a}) were purchased from BD PharMingen, (San Diego, CA), and anti-B7-H1 mAb (rat IgG_{2a}) from eBioscience (San Diego, CA). Intracellular Foxp3 staining was performed using a staining kit (eBioscience). Appropriate isotype control antibodies were used in all experiments. For CFSE labeling, T cells (10⁷/ml) were incubated with 1.25 µM CFSE (Invitrogen, Carlsbad, CA) from a 10 mM stock solution (in DMSO) for 10 min at 37°C. Cells were analyzed by an EPICS ELITE flow cytometer (Coulter Corporation, Hialeah, FL).

T cell proliferation and suppression assays

For proliferation assay, nylon-wool-eluted spleen T cells (BALB/c, 2×10^5 /well in 100 μ l) were cultured in RPMI complete medium with graded numbers of γ -irradiated (20Gy) stimulator cells from B6 mice in triplicate in 96-well plates (BD Falcon, San Jose, CA) for 3 days in 5% CO₂ in air. [³H]TdR (1 μ Ci/well) was added for the final 18 hours of culture. Cytokine levels in the culture supernatants were determined by ELISA or cytometric beads assay (CBA) kit (BD PharMingen). For suppression assays, the test cells (CD4⁺CD25⁺ or CD4⁺CD25⁻ cells) were purified using magnetic beads coated with anti-CD25 mAb at a purity of >99% (flow cytometry), and added to cultures of CFSE-labeled reporter T cells stimulated either by allogeneic spleen cells (B6) or third party spleen cells (C3H) at a suppressor:reporter:APC ratio of 1:1:1. Reporter T cell proliferation was determined by CFSE dilution.

Cytotoxic T lymphocyte (CTL) assay

BALB/c spleen T cells cultured with γ -irradiated (20Gy) B6 DC (10:1) for 5 days were used as effectors. EL4 (H2^b), P815 (H2^d) or R1.1 (H2^k) lymphoma cells (4×10^6 , ATCC, Rockville, MD) labeled with 100 μ Ci Na₂⁵¹CrO₄ (NEN, Boston, MA) were used as donor, syngeneic and third party-specific targets, respectively. Cells (5×10^3) were plated in 96-well round-bottom culture plates. Serial, two-fold dilutions of effector cells were added, and incubated for 4 hours at 37°C in 5% CO₂ in air. The results are expressed as means $\pm$ 1SD of percentage-specific ⁵¹Cr release in triplicate cultures.

RNase Protection Assay

Total RNA was extracted from cells with TRI reagent (Sigma). mRNA expression was determined using the RiboQuant multi-probe RNase protection assay system (BD PharMingen) as instructed. The murine L32 and GAPDH riboprobes were used as controls.

Semi-quantitative polymerase chain reaction (PCR)

0.2 μ g of RNA was reverse transcribed into cDNA by polymerase chain reaction (PCR) amplification using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). To obtain semi-quantitative PCR data, cDNAs were PCR amplified with 40 cycles to obtain logarithmic amplification. Fifteen μ l of each reaction product was separated on a 5% polyacrylamide gel, stained with SYBR Green I (Sigma), and scanned by Amersham Storm 860 (Amersham Biosciences, Piscataway, NJ).

Quantitative (q) PCR

mRNA levels of TLR4, IL-12-p35 and -p40, IL-10, Foxp3, CCR3, CCR5, T-bet, IFN- γ R, IL-10R, IL-27-p28 and -Ebi-3 were determined by quantitative PCR using LightCycler RNA Master SYBR Green I Kit (Roche, Indianapolis, IN). Total RNA was purified using TRIzol reagent (Invitrogen). A 1.0- μ g aliquot of RNA was treated with deoxyribonuclease I (Invitrogen), and quantitative real-time PCR performed on a Prism 7500 PCR machine (Applied Biosystems). Threshold cycle numbers were determined using a standard curve. Samples were run in duplicate and expression levels were normalized to expression of 18S or β -actin.

Statistics

The parametric data were analyzed by Student's *t* test. Values of *p*<0.05 were considered statistically significant.

Results

In vivo expansion of liver DC by tail vein injection of plasmid-GM-CSF

Studies on liver DC have lagged behind significantly due to difficulties in obtaining sufficient cells (16). We have previously used systemic administration of fms-like tyrosine kinase 3 ligand (Flt3L) for expansion, but significantly high numbers of plasmacytoid (p) DC are generated and require further separation. In addition, Flt3L-expanded DC are markedly activated and display a mature phenotype and immunostimulatory activity (17,18). We developed an approach of hydrodynamic injection of plasmid-GM-CSF. The average yield of liver mononucleocytes was 4.1×10^6 /liver in non-treated controls (8% were CD11c⁺) vs. 239.9×10^6 /liver (13% were CD11c⁺) in plasmid-GM-CSF treated mice. The mononucleocytes and CD11c⁺ cells increased by ~40 and 95 fold, respectively (Fig. 1A). Among the CD11c⁺ population, ~50% were CD11b⁺ cells in the non-treated liver, whereas ~96% were CD11b⁺ cells in plasmid-GM-CSF treated group. It has been shown that 60–80% of CD11c⁺CD11b⁺ cells in the liver are plasmacytoid (p) DC (19), indicating that most of the CD11c⁺ cells expanded with the plasmid-GM-CSF treatment were CD11c⁺CD11b⁺ myeloid DC. This was confirmed by B220 staining showing only 2.6% pDC (B220⁺) in the CD11c⁺ population (Fig. 1A). To determine that GM-CSF overexpression did not affect the phenotype and function of liver myeloid DC, myeloid DC (CD11c⁺CD11b⁺) in non-treated liver were obtained through flow sorting, and compared with bead-purified CD11c⁺ cells from the plasmid-GM-CSF treated liver. There were no significant differences in expression of MHC class II or costimulatory molecules (Fig. 1B), as well as in induction of similar degree of proliferative response in allogeneic T cells (Fig. 1C), demonstrating that plasmid GM-CSF treatment did not grossly affect the phenotype and function of liver DC. In the following studies, the liver DC (CD11c⁺) isolated from the plasmid-GM-CSF-treated mice were used.

The effect of LPS stimulation on DC surface molecule expression

DC isolated from the liver and spleen were examined for expression of TLR1-9 mRNA by semi-qPCR. Both populations were found to express all TLRs tested. Liver DC showed relatively higher expression of TLR4 compared to spleen DC, as determined by RT-PCR (Fig. 2A) and confirmed by qPCR (Fig. 2B). Further, expression of key surface molecules on DC was analyzed after exposure to LPS at a low (0.1 µg/ml) or high concentration (2.5 µg/ml). LPS at low concentration significantly enhanced expression of CD40, CD80 and CD86 on spleen DC. However, the liver DC showed altered responsiveness and upregulated expression of B7H1 only. CD40 and CD86 expressions were upregulated on liver DC only when LPS was increased to 2.5 µg/ml (Fig. 2C), suggesting that the threshold of liver DC responsiveness to LPS is higher than for spleen DC. For activation of liver DC, 2.5 µg/ml LPS was used in following experiments, unless otherwise indicated.

Liver DC respond to LPS by secreting IL-27, but not bioactive IL-12

In addition to surface molecules, cytokines produced by DC also play a critical role in T cell activation and differentiation. We assessed the cytokine mRNA expression in spleen and liver DC using RNA protection assay (RPA) or qPCR, and cytokine protein levels in DC culture supernatant by CBA or ELISA. LPS stimulated spleen DC predominantly produced IL-12 (Fig. 2D, RPA, qPCR and CBA) and IL-6 (Fig. 2D, RPA). In contrast, LPS stimulation only modestly increased expression of IL-12 p35 in liver DC, but not p40 (Fig. 2D, RPA and qPCR), consistent with our observation on low production of bioactive IL-12 p70 (Fig. 2D, CBA). Interestingly, LPS-stimulated liver DC produced large amounts of IL-27 (Fig. 2D, ELISA), a heterodimeric (namely p28 and Ebi3) cytokine of IL-12 family. qPCR revealed that IL-27-Ebi-3 was constitutively expressed in both spleen and liver DC. However, IL-27p28 was only expressed in liver DC and its expression was markedly upregulated upon LPS stimulation (Fig. 2D, qPCR). In addition, LPS also markedly enhanced expression of IL-1, IL-6, TNF-α and IL-10

in liver DC (Fig. 2D, RPA and CBA). These data indicate that cytokine production in response to LPS stimulation in spleen and liver DC is differentially regulated.

LPS-stimulated liver DC demonstrate less allostimulatory activity compared to spleen DC

The antigen presenting capacities of spleen DC and liver DC were examined in a one-way allogeneic MLR assay. As expected, spleen DC elicited vigorous proliferation of allogeneic T cells, which was further augmented by DC exposure to LPS (Fig. 3A, $p < 0.05$). Consistent with previous reports (9), liver DC elicited low T cell proliferative response. Though a statistically significant enhancement in T cell proliferation after liver DC exposure to LPS was observed, it was markedly lower than LPS-spleen DC (Fig. 3A, $p < 0.05$). The proliferative responses correlated with the generation of CTL activity, i.e. T cells stimulated by LPS-liver DC demonstrated less specific cytotoxic activity compared to those stimulated by LPS-spleen DC (Fig. 3B, $p < 0.05$). T cells stimulated by LPS-spleen DC secreted high levels of IL-2 and IFN- γ , suggesting a Th1 skewing, while T cells stimulated by LPS-liver DC produced IL-10, instead of IL-2 and IFN- γ (Fig. 3C). These trends were confirmed by analysis of other related Th1, Th2, Treg molecular markers (20). Expression of Th1-related CCR5, T-bet and IFN- γ R was greatly increased in T cells stimulated by LPS-spleen DC, whereas, T cells stimulated by LPS-liver DC expressed low CCR5 and T-bet, and high IL-10R and Foxp3, suggesting that LPS-liver DC may enhance Treg cell activity (21). No CCR3 mRNA was detected in T cells stimulated by either LPS-spleen DC or LPS-liver DC, indicating that lack of differentiation towards a Th2 subset (Fig. 3D) (20).

LPS-liver DC expand more CD25⁺Foxp3⁺ cells

To obtain direct evidence that LPS-liver DC are capable of facilitating expansion of Treg cells, CFSE-labeled spleen T cells from BALB/c mice were cultured with γ -irradiated B6 spleen DC or liver DC (with or without stimulation by LPS). Cells were then stained with mAbs against CD4, CD25 and Foxp3 for flow analysis. Naive CD4⁺ T cells contained ~6.5% of CD25⁺FoxP3⁺ cells, reflecting the “naturally-existing” Treg cell population (22). Frequency of CD25⁺FoxP3⁺ cells in T cells cultured alone for 5 days dropped to 1.2% (data not shown), presumably due to lack of required stimulation to maintain Treg cell survival. Spleen DC stimulated allogeneic T cells to generate predominantly CD25⁺Foxp3⁺ cells. This tendency was markedly enhanced by spleen DC exposure to LPS (Fig. 4A), and was associated with enhanced IL-2 and IFN- γ production (Fig. 3C), indicating a Th1 skewed differentiation. On the other hand, the CD4⁺CD25⁺ cells expanded by liver DC were predominantly FoxP3⁺. CFSE dilution analysis showed that the CD25⁺FoxP3⁺ cells expanded by liver DC or LPS-liver DC were proliferative (Fig. 4A). These data indicate that liver DC stimulate allogeneic T cells towards a greater expansion of CD4⁺CD25⁺FoxP3⁺ cells, an effect that can be further enhanced by LPS stimulation. The expansion of CD25⁺FoxP3⁺ cells was most likely independent on B7-H1, since expansion of CD25⁺FoxP3⁺ by B7-H1 deficient LPS-liver DC was found to be comparable to WT controls (Fig. 4A).

Immune suppressive effect of CD4⁺CD25⁺ cells induced by LPS-liver DC

Functional CD25⁺FoxP3⁺ cells could not be isolated by flow sorting due to requirement for cell membrane permeabilization during intracellular staining for Foxp3. An alternative approach was to examine the CD4⁺CD25⁺ population, in which 82% of cells were found to be FoxP3⁺ following LPS-liver DC stimulation (Fig. 4A). CD4⁺CD25⁺ or CD4⁺CD25⁻ cells were purified from cultures of BALB/c T cells with irradiated B6 LPS-spleen DC or LPS-liver DC, and added to CFSE-labeled BALB/c T cell culture (reporter cells). Reporter T cell proliferation was elicited by irradiated B6 spleen cells and determined by CFSE dilution assay. We demonstrated that the CD4⁺CD25⁺ cells generated by LPS-liver DC, but not those generated by LPS-spleen DC group, markedly inhibited proliferative responses of reporter cells (Fig.

4B), indicating existence of Treg cell activity. To determine whether the inhibition was MHC specific, BALB/c CD4⁺CD25⁺ cells driven by B6 (H-2^b) LPS-liver DC were added to CFSE-labeled BALB/c T cells stimulated by spleen cells from third party strain (C3H, H-2^k). A comparable inhibition of the proliferative response was also seen in T cells stimulated by third party antigens (Fig. 4B), suggesting that the inhibitory effect of LPS-liver DC-induced Treg cells is not MHC-specific. As expected, CD4⁺CD25⁺ cells from LPS-spleen DC group did not show any inhibitory effects on T cell proliferative response (Fig. 4B).

IL-27 secreted by liver DC is crucial in induction of T cell hyporesponsiveness

To address whether liver DC-induced T cell hyporesponsiveness was due to lack of IL-12 production, exogenous IL-12 was added into the MLR culture. T cell proliferative response was enhanced (Fig. 5, $p < 0.05$) albeit slightly, when the exogenous IL-12 was increased to 4ng/ml. Interestingly, abrogation of IL-27 ligation by using T cells from IL-27R^{-/-} mice synergistically augmented the effect of exogenous IL-12 (Fig. 5, $p < 0.05$, IL-27^{-/-} + IL-12 group vs. WT + IL-12 group). Elimination of IL-27 ligation alone showed no effect (Fig. 5, $p > 0.05$, IL-27^{-/-} group vs. WT group), suggesting that IL-27 produced by liver DC plays a crucial role in induction of T cell hypo responsiveness via counteracting the effect of IL-12, a potent Th1 stimulatory factor (23). Our data indicated that liver DC appeared to respond to LPS by secreting IL-27, but not bioactive IL-12, which profoundly inhibits T cell responses.

Discussion

We demonstrated that both spleen and liver DC expressed all TLR1-9 mRNAs, suggesting that they are well equipped to receive a wide range of innate immune signals. This is consistent with a recent report that DC have the capacity to elicit immune responses to a variety of pathogens (24). We focused on TLR4, because it is expressed at higher levels in liver DC (Fig. 2A). Our study emphasizes the differences between liver DC and conventional spleen DC in response to LPS stimulation. Liver DC exhibit endotoxin tolerance by requiring markedly higher concentration of LPS for activation compared to conventional DC. Endotoxin tolerance was initially described 50 years ago as a reduced capacity of the host cell to respond to LPS activation following first time exposure to this stimulus (25). Endotoxin tolerance of liver DC may reflect an adaptive property as they are constantly exposed to bacterial products from the gut. DC in the liver maintain a high threshold for triggering TLR4 signaling, resulting in preventing themselves from being over-activated.

The precise mechanisms of the distinct response to endotoxin by liver DC remain unclear. Low expression of TLR4 is an unlikely explanation given that the expression of TLR4 mRNA in liver DC was even higher than spleen DC in our experimental setting. This is contrary to previous reports showing that liver DC isolated from mice following systemic treatment with Flt3 ligand expressed low TLR4 (26). The differences in observation might reflect deviations of liver DC in response to treatment with GM-CSF and Flt3 ligand. We demonstrated that liver DC become phenotypically mature following high concentration LPS stimulation and retain the capacity to induce T cell hyporesponsiveness and expand CD4⁺Foxp3⁺ Treg cells. Our data are consistent with a recent report showing that fully mature DC can expand Treg cells and induce T cell hyporesponsiveness (8).

The results in this study provide evidence that following LPS stimulation, fully mature liver DC produced large amounts of IL-27 and IL-10, instead of bioactive IL-12. Induction of T cell hyporesponsiveness by LPS-liver DC was not simply due to a lack of IL-12 because addition of exogenous IL-12 could only modestly enhance T cell proliferation. We noted that abrogation of IL-27 signaling by using T cells from IL-27R^{-/-} mice synergistically augmented the effect of IL-12, suggesting that IL-27 produced by liver DC plays a crucial role in induction of T cell hyporesponsiveness by counteracting the effect of IL-12. This study showed that Ebi-3 subunit

of IL-27 was constitutively expressed in both spleen and liver DC, and was modestly upregulated following LPS stimulation. However, p28 was only expressed in liver DC and its expression was markedly increased by TLR4 ligation, suggesting that IL-27 in liver DC may be regulated by expression of p28. This is in agreement with several recent reports showing that expression of p28 affects function of myeloid DC (27). IL-27 shares homology with IL-12, and has complex role in the control of innate and adaptive immune responses (23). It was first recognized as a proinflammatory cytokine with Th1-inducing activity. Subsequent work demonstrated that a deficiency in IL-27 receptor showed exacerbated inflammatory responses, suggesting that IL-27 has an immunoregulatory function (28). Recent studies have shown that IL-27 can activate naïve T cells, however during later phases, IL-27 suppressed IFN- γ and IL-17 production in Th1 and Th17 cells respectively (29,30). In current study it was noticed that liver DC also produce the inflammatory cytokines IL-1 α , IL-6 and TNF- α , the effects of which might be overcome by IL-27 suppressive activity. It is interesting to know whether liver DC influence Treg cell responses also as these cells express the IL-27R complex (31,32). Our results revealed that LPS-activated IL-27-producing liver DC preferentially expanded CD25⁺Foxp3⁺ Treg cells, whereas, LPS-spleen-DC that secreted IL-12 predominantly promoted generation of Th1 cells. The distinct response to LPS by liver DC may be an important mechanism by which immune responses in the liver are modulated via bridging of innate and acquired immunity.

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Abbreviation

APC	antigen-presenting cells
DC	dendritic cells
Flt3L	fms-like tyrosine kinase 3 ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
IL	interleukin
IFN	interferon
NPC	nonparenchymal cell
MHC	major histocompatibility complex
LPS	Lipopolysaccharide
PCR	polymerase chain reaction
Treg	regulatory T (cell)
TLR	toll-like receptor

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Figure 1. Plasmid-GM-CSF expanded liver DC exhibit normal phenotype and allostimulatory function

(A) Purification of liver myeloid DC. Mononucleocytes isolated from the liver of plasmid-GM-CSF injected B6 mice (normal B6 mice serve for comparison) as described in Methods were stained with anti-CD11b and -CD11c, and analyzed by flow cytometry. Myeloid liver DC in non-treated mice were purified by flow sorting of CD11b⁺ CD11c⁺ population, while myeloid DC in plasmid-GM-CSF treated group were simply purified by CD11c⁺ beads selection. The purity and content of pDC (B220⁺) in the CD11c population are shown in the histogram plots. (B) Expression of MHC class II and costimulatory molecules on myeloid liver DC. Liver myeloid DC from plasmid-GM-CSF treated and from non-treated mice were stained by mAbs against listed molecules and showed very similar profile. (C) Impact of plasmid-GM-CSF treatment on allostimulatory activity of liver DC. In a one-way MLR culture, BALB/c spleen T cells (2×10^5 /well) as responders were cultured for 3 days with γ -irradiated stimulators from B6 mice in graded numbers: liver DC from plasmid-GM-CSF treated (B6 GM-CSF LDC), liver or spleen DC from non-treated (B6 LDC and B6 SDC), or spleen cells from non-treated mice (B6 spleen cells). Spleen cells from non-treated BALB/c mice were used as syngeneic

controls. T cell proliferative response was determined by thymidin uptake, and expressed as mean cpm $\pm$ 1SD. The data are representatives of two separate experiments.

(C)

Figure 2. Effect of LPS stimulation on key molecule and cytokine expression in liver DC
 (A) Expression of TLR1 to 9 mRNA in spleen and liver DC. Total RNA was isolated from liver or spleen DC, and expression of TLR was measured by a semi-quantitative PCR assay.
 (B) TLR4 mRNA in spleen and liver DC was quantified by qPCR.
 (C) Expression of cell surface molecules was determined by flow cytometry following staining with specific mAb (filled histograms). The isotype controls are plotted as empty histograms. For LPS stimulation, liver or spleen DC were incubated with LPS at low (0.1 $\mu\text{g/ml}$) or high concentration (2.5 $\mu\text{g/ml}$) for 18 hours.
 (D) Cytokines mRNA in spleen or liver DC with or without LPS stimulation was measured by RNase protection Assay (RPA) and qPCR. The protein levels of cytokines were measured following culture of liver or spleen DC for 18 hours, and the cytokines in supernatant were assessed by cytometric beads assay (CBA) or ELISA. The data are representative of two separate experiments.

Figure 3. Influence of TLR4 ligation on allostimulatory activity of liver DC

(A) In a one way MLR assay, spleen T cells (2×10^5) from BALB/c (H2^d) mice were cultured for 3 days with graded number of irradiated (20 Gy) B6 (H2^b) spleen DC or liver DC with or without stimulation of LPS (2.5 μ g/ml). Results were expressed as mean cpm of [³H]TdR incorporation $\pm$ 1SD in triplicate cultures. (B) In a CTL generation assay, BALB/c spleen T cells cultured with γ -irradiated B6 spleen or liver DC with or without LPS stimulation (T:DC ratio of 10:1) for 5 days were used as effector cells. Cytotoxicity to EL4 (H2^b) targets was determined at various effector : target cell ratios in a 4 hour ⁵¹Cr release assay. No cytotoxicity was generated against syngeneic P815 (H2^d) or third party R1.1 (H2^k) targets (data not shown). The results are expressed as means $\pm$ SD of percentage-specific ⁵¹Cr release in triplicate

cultures. (C) BALB/c spleen T cells that were cultured with irradiated B6 spleen or liver DC with or without stimulation of LPS a T:DC ratio of 10:1 for 3 days. IL-2 and IFN- γ levels in the culture supernatants of were measured by CBA. Total RNA was isolated from T cells, and mRNA for CCR5, T-bet, CCR3, Foxp3 and IL-10R was determined by qPCR.

Figure 4. Effect of LPS ligation on the capacity of spleen and liver DC to expand CD4⁺CD25⁺FoxP3⁺ cells

(A) Selective expansion of CD4⁺CD25⁺FoxP3⁺ cells by LPS-liver DC. CFSE labeled T cells (2×10^5) purified from BALB/c spleen cells were cultured with irradiated B6 spleen or liver DC with or without LPS simulation or LPS stimulated liver DC from B7-H1 knockout mice at a ratio of 10:1 for 5 days. At termination of culture, T cells were harvested and triple stained with mAbs against CD4, CD25 and FoxP3. FACS analysis was performed on the CD4⁺ population. Cell proliferation was determined by CFSE dilution assay in gated CD25⁺Foxp3⁺ cells (Upper panels). Freshly isolated T cells alone or cultured for 5 days were used as controls. Appropriate isotype control Abs were used in the experiment. (B) LPS-liver DC-expanded CD4⁺CD25⁺ cells inhibit T cell proliferative responses. CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells purified, using magnetic bead-coated anti CD25 mAb, from a culture (5 days) of BALB/c CD4⁺ T cells with B6 DC were used as the suppressors, and added into a MLR culture of CFSE labeled reporter cells [BALB/c spleen T cells whose proliferation was

elicited by irradiated B6 spleen cells (APC)] with a ratio of suppressor:reporter:APC at 1:1:1. Inhibitory activity against reporter T cell proliferation was determined by CFSE dilution analysis. To determine MHC specificity of inhibition, BALB/c CD4⁺CD25⁺ cells driven by B6 LPS-liver DC were added into CFSE-labeled BALB/c T cells whose proliferation was elicited by spleen cells from third party strain (C3H). The data are representative of two separate experiments.

Figure 5. T cell hyporesponsiveness induced by LPS-liver DC is mediated by IL-27

In a one-way MLR assay, spleen T cells (2×10^5) from wild type B6 (H2^b) or IL-27R^{-/-} (B6 background) mice were cultured for 3 days with irradiated (20 Gy), LPS-stimulated BALB/C (H2^d) liver DC (2×10^4) in the presence or absence of exogenous IL-12 (4 ng/ml). Results were expressed as mean cpm of [³H]TdR incorporation $\pm$ 1SD in triplicate cultures. The data are representative of three separated experiments.