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Poor allostimulatory function of liver plasmacytoid DC is associated with pro-apoptotic activity, dependent on regulatory T cells

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Abstract

Background/Aims—The liver is comparatively rich in plasmacytoid (p) dendritic cells (DC),-innate immune effector cells that are also thought to play key roles in the induction and regulation of adaptive immunity.

Methods—Liver and spleen pDC were purified from fms-like tyrosine kinase ligand-reated control or lipopolysaccharide-injected C57BL/10 mice. Flow cytometric and molecular biologic assays were used to characterize their function and interaction with naturally-occurring regulatory T cells (Treg).

Results—While IL-10 production was greater for freshly-isolated liver compared with splenic pDC, the former produced less bioactive IL-12p70. Moreover, liver pDC expressed a low Delta4/Jagged1 Notch ligand ratio, skewed towards T helper 2 cell differentiation/cytokine production, and promoted allogeneic $CD4^+$ T cell apoptosis. T cell proliferation in response to liver pDC was, however, enhanced by blocking IL-10 function at the initiation of cultures. In the absence of naturally occurring $CD4^+CD25^+$ regulatory T cells, similar levels of T cell proliferation were induced by liver and spleen pDC and the pro-apoptotic activity of liver pDC was reversed.

Conclusion—The inferior T cell allostimulatory activity of *in vivo*-stimulated liver pDC may depend on the presence and function of Treg, a property that may contribute to inherent liver tolerogenicity.

Keywords

Dendritic cells; Liver; T cells; Mouse; Endotoxin; Toll-like receptor; Tolerance

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4Abbreviations: DC, dendritic cells; Flt3L, fms-like tyrosine kinase 3 ligand; mDC, myeloid DC; pDC plasmacytoid DC; Treg, regulatory T cells; Foxp3, forkhead winged helix protein-3

1. Introduction

It is generally accepted that the liver has inherent tolerogenic properties (1,2), as evidenced by its role in oral and portal venous tolerance and the comparative ease of acceptance of hepatic allografts in animals (3–5) and humans (6–8). Dendritic cells (DC) are highly-specialised initiators and regulators of innate and adaptive immunity, that have been implicated in hepatic tolerogenicity (2,9,10). Impairment of their function also contributes to immune suppression during sepsis (11). While interstitial DC are rare in normal liver, making them difficult to isolate (12), their numbers are increased following partial hepatectomy (13) and enhanced by endogenous hematopoietic growth factors, in particular fms-like tyrosine kinase-3 ligand (Flt3L) (14,15).

Several DC subsets are present in normal mouse liver (16), including conventional myeloid (m)DC ($CD11c^+CD11b^+CD8\alpha^-$), $CD8\alpha^+$ DC ($CD11c^{lo}CD11b^-$), natural killer (NK) DC and plasmacytoid (p)DC ($CD11c^-CD11b^-NK1.1^-B220^+$) (17–20). pDC are more abundant in the liver compared with secondary lymphoid tissue, such as spleen (19). They are the major source of type-1 interferons (IFNs) in the body (21) and important in anti-viral responses, although their numbers and function are reduced in hepatitis C virus-infected livers (22). While mDC tend to prime T helper (Th)1 responses, pDC can induce Th1, Th2 or T regulatory cell (Treg) responses, depending on the nature of the antigen (Ag) and the costimulatory signals they transmit to T cells (23,24). Both mDC and pDC subsets, particularly immature DC, can exhibit tolerogenic properties and expand or induce regulatory T cells (Treg) (25). Recently, pDC have been implicated in transplant tolerance and the induction of Ag-specific Treg in allograft recipients (26,27).

Since the liver is located downstream from the gut, interstitial DC and other hepatic APC are exposed continually to microbe-derived ‘danger’ signals, recognized by pattern-recognition receptors, in particular Toll-like receptors (TLR) (28,29). Refractory responses of freshly-isolated murine liver DC (bulk DC or mDC) to TLR stimulation (‘endotoxin tolerance’) (30–32) may represent an adaptive response to prevent chronic liver inflammation. The mechanistic basis of endotoxin tolerance has been studied largely in macrophages, and negative regulators of TLR signaling have been implicated in promotion of this acquired hyporesponsiveness (33,34). Little work has been conducted on DC, in particular liver DC, although recently, IL-6 and signal transducer and activator of transcription 3 activity have been shown to down-regulate responses of murine liver DC to endotoxin (32).

DC that are phenotypically immature, such as freshly-isolated liver DC (17,19,30), and mDC that are maturation-resistant (35), are poor T cell stimulators and can regulate alloimmune reactivity, in vitro and *in vivo*. On the other hand, TLR4 engagement on hepatic Ag-presenting cells (APC) is involved in liver inflammation (36,37). Since mouse pDC [that constitutively express low levels of TLR4 (30,38–40)] are well-represented in the liver, the influence of TLR4 ligation on their function and on the outcome of their interactions with T cells may impact significantly on hepatic immune reactivity and alloimmune responses. Indeed, intrahepatic lipopolysaccharide (LPS) levels may be elevated intraoperatively in liver transplantation (41). In this study, we examined the functional biology of liver pDC from control and LPS-stimulated mice. We also investigated mechanisms underlying differences between liver and splenic pDC in allogeneic T cell stimulatory ability, including the role of naturally-occurring Treg. The findings confirm that, compared with spleen pDC, liver pDC are inferior allostimulators and resistant to LPS stimulation *in vivo*. Moreover, their depressed ability to stimulate T cell proliferation, and their capacity to promote T cell apoptosis appear to be dependent on the presence of Treg.

2. Materials and methods

2.1 Mice

Male C57BL/10 (B10) ($H2^b$) and BALB/c ($H2^d$) mice (8–12 wks old) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free facility of University of Pittsburgh School of Medicine. Experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and under an Institutional Animal Care and Use Committee-approved protocol. Mice received Purina rodent chow (Ralston Purina, St. Louis, MO) and tap water ad libitum.

2.2 Reagents

RPMI-1640 complete medium was used for cell culture (31). Chinese hamster ovary cell-derived recombinant human fms-like tyrosine kinase 3 ligand (Flt3L) was provided by Amgen (Seattle, WA). LPS (ultra pure; *E.coli* K12) was purchased from Invivogen (San Diego, CA). Mice received LPS (100 μg) or PBS i.v. via the lateral tail vein. Neutralizing rat anti-mouse IL-10 mAb (JES5-2A5; azide-free) was from BD PharMingen (San Diego, CA). Recombinant mouse IL-12 was from Sigma (St. Louis, Mo).

2.3 Isolation of DC

$CD11c^+$ cells were isolated from livers and spleens of normal animals or mice given the DC poietin Flt3L (10 μg/mouse/day i.p., for 10 days). Bulk DC were enriched by density centrifugation using Nycodenz (Sigma). For pDC purification (>95%), $mPDCA1^+$ cells were positively selected from the DC-enriched fraction using immunomagnetic beads (Miltenyi Biotec, Auburn, CA). For mDC purification, cells harvested after density centrifugation were incubated with biotin-conjugated rat anti-mouse B220/CD45R and CD49b and depleted by negative selection. $CD11c^+$ cells were then positively selected using MACS® (Miltenyi Biotec). The purity of mDC ($CD11c^+B220^-DX\text{-}5^-$) was consistently >95 % (35).

2.4 Flow cytometry (cell surface staining)

Cells were treated with FcγR-blocking Ab (rat anti-mouse CD16/32 mAb) (2.4G2) to avoid non-specific Ab binding. For cell surface staining, they were then incubated for 30 min with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-Cy5, or PE-Cy7-conjugated mAbs to detect expression of CD11c (clone HL3), B220/CD45R (RA3-6B2), IA^b β-chain (25-9-17), CD86 (GL1), TLR4 (UT41) (eBioscience, San Diego, CA) or FasL (CD95L) (MFL3) (eBioscience). These mAbs, and appropriate Ig isotype controls, were obtained from BD PharMingen, unless specified. Flow analysis was performed using a LSR II flow cytometer (BD Bioscience, San Jose, CA) and results expressed as % positive cells and mean fluorescence intensity (MFI).

2.5 Real-time RT-PCR

Messenger RNAs (mRNAs) for TLR4, Delta4, Jagged1 and β actin were quantified in duplicate by SYBR Green two-step, real-time RT-PCR. After generating first-strand cDNA from purified DCs, the PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA), with primers designed according to published sequences (42, 43). Thermal cycling conditions were 10 min at 95°C to activate DNA polymerase, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Using the manufacturer's software, real-time PCR data were plotted as the ΔR_n fluorescence signal versus the cycle number. The threshold cycle was defined as the cycle number at which the ΔR_n crossed this threshold. The expression of each gene was normalized to β actin mRNA content and calculated relative to control using the comparative cycle threshold method.

2.6 T cell purification

$CD4^+$ T cells were purified from spleen cell suspensions using Dynal® Mouse CD4 Negative Isolation kits (Dynal Biotech, Oslo, Norway). In some experiments, the resultant $CD4^+$ population was then incubated with biotin-conjugated anti-CD25 mAb (PC61-5; eBioscience) to remove naturally-occurring Treg, and $CD4^+CD25^-$ T cells isolated by negative selection using anti-biotin microbeads and LD separation columns (Miltenyi Biotec) (35,44). Purity was consistently >97%.

2.7 Mixed leukocyte reaction (MLR)

MLRs were performed using graded numbers of B10 DC as stimulators of purified allogeneic BALB/c T cells (2×10^5/well) in 72 h MLR using 96-well, round-bottom plates, as described (45). In some experiments, neutralizing anti-IL-10 mAb, isotype control Ig or IL-12 was added at the start of cultures.

2.8 CFSE-T cell proliferation assay

Purified $CD4^+$ T cells were CFSE (carboxyfluorescein succinimidyl ester)-labeled using the Vibrant CFDA SE Cell Tracer Kit (Invitrogen) according to the manufacturer's instructions. BALB/c bulk $CD4^+$ or $CD4^+CD25^-$ T cells (2×10^5) were co-cultured with 1×10^4 allogeneic (B10) DC for 4 days in 96-well, round-bottom plates, then analyzed by flow cytometry.

2.9 Analysis of T cell apoptosis

$CD4^+$ or $CD4^+CD25^-$ T cells stimulated with DC in MLR at a 20:1 ratio were harvested at 72h and labeled with FITC-conjugated anti-CD4 mAb and PE-Cy5-conjugated anti-CD11c mAb. The incidences of viable cells and early and late apoptotic T cells were determined using Annexin V-PE apoptosis detection kits (BD PharMingen). After staining of externalized phosphatidylserine with Annexin-V-PE and incubation in the vital dye 7-amino-actinomycin D (7-AAD), data were acquired immediately and analyzed as described above.

2.10 Intracellular cytokine staining

Purified DC were treated with brefeldin A (GolgiPlug™; 1 μl/ml, BD PharMingen) for 16 h, then labeled with FITC- or PE-Cy5-conjugated mAb and fixed in 1% paraformaldehyde. The cells were permeabilized with 0.1% saponin, then incubated with anti-IL-12p40/p70 (C15.6) and anti-IL-10 (JES5-16E3), or rat IgG (all BD PharMingen) for 30 min. T cells recovered from 96 h MLR were restimulated with plate-bound anti-CD3ε (10 μg/ml; clone 17A2) and 2 μg/ml soluble anti-CD28 mAb (37.51) (each from BD PharMingen) for 5 h at 37°C, in the presence of brefeldin A. After cell surface staining with PE-Cy7-CD4 mAb, the cells were fixed, permeabilized, then stained with anti-IFNγ (XMG1.2), anti-IL-4 (BVD4-1D11) or isotype control Ig (all from BD PharMingen).

2.11 ELISA

Levels of IL-12p70, IFN , IL-4, or IL-10 in culture supernatants were determined by ELISA, using commercial kits (Biolegend Inc., San Diego, CA) and following the manufacturer's instructions.

Statistical analyses—Data are expressed as means ± 1 SD. Significances of differences between means were determined by unpaired Student's 't'-test. A 'p' value < 0.05 was considered significant.

3. Results

3.1 Freshly-isolated liver pDC from control and LPS-injected mice induce less allogeneic T cell proliferation than splenic pDC

To examine their allostimulatory activity, freshly-isolated pDC (and for comparison, mDC) from livers or spleens of control (B10) mice or animals injected 2 hr earlier with LPS, were co-cultured in MLR with normal, allogeneic (BALB/c) $CD4^+$ T cells. T cell proliferation was quantified by both [^{3}H]TdR incorporation and CFSE dilution analysis, as described in the Materials and methods. pDC elicited lower levels of T cell proliferation than mDC (Fig. 1A), especially after *in vivo* LPS administration. Compared with spleen pDC, liver pDC from LPS-injected mice were poor stimulators of $CD4^+$ T cell proliferation (Fig. 1A, B). To determine whether the difference in allostimulatory capacity between liver and spleen pDC was due to a direct LPS effect, mPDCA-1^+ cells from both tissues were cultured with LPS (1 μg/ml) overnight (16h). LPS-stimulated liver pDC induced significantly less T cell proliferation than LPS-stimulated spleen pDC of Flt3L-treated animals (Fig. 1C). Further, liver pDC from LPS-injected normal (non-Flt3L-treated) mice displayed similar, significantly reduced ability to induce proliferation of allogeneic CD4+ T cells compared with normal LPS-activated spleen pDC (Fig. 1D).

3.2 MHC class II, CD86 and TLR4 expression do not differ significantly between liver and spleen pDC

To determine whether liver pDC were refractory to LPS-induced maturation, we analyzed MHC class II and costimulatory molecule (CD86) expression on freshly-isolated liver and spleen pDC from LPS-infused B10 mice by flow cytometry. Liver and spleen pDC from control mice expressed similar, low to intermediate levels of surface MHC class II (IA^b) and CD86. Following LPS administration, MHC class II and CD86 were upregulated significantly on spleen pDC, but only the incidence of $CD86^+$ cells was upregulated significantly for liver pDC (Fig.2A). TLR4 message and cell surface expression of TLR4 did not differ significantly between freshly-isolated, immunobead-purified liver and spleen pDC (mPDCA-1^+), as determined by quantitative RT-PCR and flow cytometry, respectively (Fig 2B & C).

3.3 LPS-stimulated liver pDC express lower levels of IL-12, but higher levels of IL-10 that regulates T cell proliferation

To gain further insight into the functional differences between liver and spleen pDC, we next examined the expression of specific immunomodulatory cytokines by these cells, using flow cytometry and ELISA. As shown in Fig. 3A, flow analysis of intracellular cytokine levels revealed similar low IL-12 (p40/p70), but much higher IL-10 levels in control and LPS-stimulated liver compared with spleen pDC. IL-12 (IL-12p70) secretion by *in vivo*-stimulated liver pDC was significantly lower than that of splenic pDC. On the other hand, IL-10 secretion by liver pDC was significantly higher than that of splenic pDC, with or without LPS administration (Fig. 3B). Thus, the inferior T cell allostimulatory ability of in vivo LPS-stimulated liver pDC compared with spleen pDC is consistent with their differential regulation of pro-(IL-12) and anti-inflammatory cytokine (IL-10) production. In keeping with this conclusion, addition of neutralizing anti-IL-10 mAb or exogenous IL-12 at the start of LPS-stimulated liver pDC:allogeneic T cell MLR, significantly enhanced the proliferative response to a level similar to that achieved with splenic pDC (Fig C & D).

3.4 LPS-stimulated liver pDC skew allogeneic T cells towards Th2 differentiation, associated with a low Delta 4/Jagged 1 ratio

We next compared intracellular Th1 (IFNγ) and Th2 cytokine (IL-4) production by normal, allogeneic $CD4^+$ T cells stimulated with liver or spleen pDC. As shown in Fig. 4A, the

incidences of IFNγ- and IL-4-producing $CD4^+$ T cells induced by control or LPS-activated pDC in MLR were lower for liver compared with splenic pDC. This resulted in a significantly higher $IL\text{-}4^+/IFN\gamma^+$ $CD4^+$ T cell ratio for liver pDC compared with spleen pDC (Fig 4B). The levels of secreted IFNγ were substantially and significantly lower in MLR cultures stimulated with liver pDC, whereas secreted levels of IL-4 did not differ significantly (data not shown).

Previous studies (46) have suggested that the balance of expression of different Notch ligands on APC may direct $CD4^+$ T cells to differentiate into either Th1 or Th2 cells. According to this paradigm, elevated expression of Delta 4 induces Th1 cells, while elevated expression of Jagged1 induces Th2 cells. Thus, the ratio of Delta 4 to Jagged1 mRNA expression may predict a Th1 response. As shown in Fig. 4C, the expression of Jagged1 relative to Delta 4 mRNA was lower on pDC from liver compared with spleen pDC of LPS-injected mice, consistent with comparatively poor Th1-polarizing function of the liver pDC, and skewing towards Th2.

3.5 The inferior T cell allostimulatory activity of liver pDC is dependent on naturally-occurring $CD4^+$ $CD25^+$ Treg

Phenotypically immature DC with weak allostimulatory capacity can expand or enrich for Treg (35,47). We therefore examined whether the poor T cell stimulatory properties of liver pDC might correlate with interactions with naturally-occurring $CD4^+CD25^+$ ($Foxp3^+$) Treg. Although CD25 is not a definitive marker of functional T reg, naturally-arising $CD25^+$ $CD4^+$ T cells play an indispensable role in self tolerance and negative control of immune responses (48). When naturally-occurring $CD4^+$ $CD25^+$ cells were removed from the normal responder T cell population, immediately before the start of MLR, similar extents of CFSE-labeled T cell proliferation were observed when liver or spleen pDC from LPS-treated mice were used as stimulators (Fig. 5A & B), reversing the inferior allostimulatory capacity of the liver pDC significantly. These data suggest that Treg are rendered more effective at inhibiting effector T cell proliferation in the presence of liver than spleen pDC, and that naturally-occurring Treg may suppress the differentiation/function of liver pDC.

3.6 LPS-stimulated liver pDC induce a higher incidence of early and late apoptosis in allogeneic $CD4^+$ T cells than splenic pDC: dependence on naturally-occurring $CD4^+CD25^+$ Treg

To further investigate mechanisms that might underlie the poor T cell proliferative response induced by liver pDC, we next examined the influence of pDC on apoptosis of normal allogeneic responder bulk $CD4^+$ T cells stimulated with liver or splenic pDC from LPS-injected mice. As shown in Fig. 6A and B (left side), the incidence of early (annexin V^+ $7AAD^-$) and late (annexin V^+/7-AAD^+) apoptotic $CD4^+$ T cells was enhanced significantly in MLR cultures stimulated with liver compared with spleen pDC. However, when naturally-occurring $CD4^+$ $CD25^+$ Treg were removed from the responder T cell population, immediately before the start of the MLR, the pro-apoptotic effect of liver pDC was reversed (Fig. 6B; right side). Similar levels of cell surface death ligand FasL (CD95L) were expressed by the pDC populations from the 2 organs (Fig. 6C), making it very unlikely that signaling via the FasL-Fas pathway contributed to the greater proapoptotic activity of liver pDC.

4. Discussion

DCs are rare, uniquely well-equipped bone-marrow-derived professional APCs, found ubiquitously in lymphoid and non-lymphoid tissues. pDCs constitute a major subset of DCs in mice and humans (49,50) and play crucial roles in innate and adaptive immunity (51). Compared with the spleen, relatively high proportions of pDC relative to conventional mDC are found in normal mouse liver, or in livers in which DC have been expanded by systemic administration of the DC poietin Flt3L (19,39). In the recent study by Shu et al (39), a ratio of

pDC to mDC in normal liver about 5 times higher than that in the spleen was reported, whereas a 4 times higher incidence of pDC relative to mDC in normal mouse liver was reported by Pillarisetty et al (19). Due to their overall paucity in normal liver, we used Flt3L as in our and others' previous studies (15,17,30,31,52,53), to expand liver DC, in particular pDC. It has been reported that Flt3L treatment (adenoviral gene delivery) for 10 days (the standard dosing schedule) increases liver pDC 200-fold, and spleen pDC 28-fold, thus enabling the isolation and purification of adequate numbers of pDC for analysis. Use of Flt3L protein to mobilize DC in this study avoided the possibility of DC activation associated with adenoviral delivery of the Flt3L gene (54).

Previous reports have shown that bulk liver DC are less mature and have weaker T cell stimulatory ability than DC from secondary lymphoid tissues (15,19,30,39). We found that both freshly-isolated normal mouse liver and spleen pDC were phenotypically immature and weak allogeneic T cell stimulators. However, compared with splenic pDC from LPS-injected mice, liver pDC were much inferior allostimulators. Moreover, liver pDC produced the anti-inflammatory cytokine IL-10, and were deficient (compared with spleen pDC) in their capacity to elicit IFNγ production by allogeneic $CD4^+$ T cells, and skewed towards Th2 differentiation. This was consistent with the low Delta 4/Jagged 1 ratio (46) observed in LPS-stimulated liver compared with spleen pDC. In addition, liver pDC promoted higher incidences of apoptotic $CD4^+$ T cells in MLR than spleen pDC. The functional deficiency of liver pDC as T cell stimulators and their proapoptotic activity was reversed by removal of $CD4^+CD25^+$ cells at the start of cultures, indicating that the deficiency was dependent on naturally-occurring Treg. Our preliminary findings also reveal that, in the presence of liver pDC compared with spleen pDC, the production of the cytotoxic effector molecule perforin by Treg is enhanced (unpublished observations), suggesting a mechanism (55) that may underlie the poor allogeneic effector T cell responses observed with liver pDC.

There have been few previous accounts of the phenotype and function of pure populations of pDC isolated from mouse or human liver. Recently, Shu et al (39) reported that liver pDC from normal mice exhibited efficient endocytotic activity, a characteristic of immature DC, and produced significant levels of IL-12p40, IL-6 and tumor necrosis factor α (IL-10 was not examined) in response to TLR agonists. However, as reported herein, these DC were much inferior inducers of allogeneic T cell proliferation than splenic pDCs. As in the present study, Kingham et al (15) used Flt3L to expand hepatic pDC; they reported that the *in vivo*-expanded liver pDC (induced by adenovirus encoding murine Flt3L cDNA) secreted similar levels of IL-12, IL-6 and IL-10, but comparatively high levels of IFNα following in vitro TLR9 ligation. However, CpG stimulation failed to enhance their poor inherent T cell allostimulatory ability. Despite differences in the models used, and in the precise nature of the investigations conducted in these and the current studies, the findings endorse overall the view that pDC, relatively enriched in the liver environment, are inferior stimulators of adaptive T cell responses.

Evidence has accumulated that pDC play an important role in regulation of T cell responses and in the promotion of tolerance (25), including the induction of Treg (24,56–58). Our finding that liver pDC have greater ability than their splenic counterparts to enhance the apoptotic death of allogeneic T cells is in keeping with similar findings on immature/maturation-resistant or regulatory DC (35,59,60). It is also consistent with the apoptotic death of graft-infiltrating T cells, that correlates with murine liver allograft survival in the absence of immunosuppressive therapy (61). Other studies have shown that pDC (*in vivo*-mobilized, as in this study) can promote allogeneic stem cell engraftment and skin graft survival (27), and inhibit organ allograft rejection (62). In addition, pDC in secondary lymphoid tissue appear to mediate tolerance to organ grafts by inducing alloAg-specific Treg (26). To our knowledge, there have been no previous reports of interactions between liver pDC and Treg. pDC are comparatively abundant in the liver (19,39), and there is also a substantial population of naturally-occurring

Treg in this organ (63). Our novel finding that naturally-occurring Treg down-modulate the ability of *in vivo*-stimulated liver pDC to drive allogeneic T proliferation to a greater extent than splenic pDC, suggests a mechanism that may underlie the deficient stimulatory function of this comparatively enriched DC subset in the liver.

It has been proposed for some time that, in the context of allograft tolerance, hepatic DC can induce T cell apoptosis and exert regulatory/suppressive functions (9,64,65). In this regard, it may be significant that T cells activated in the liver are functionally defective, with shortened half-life (66). The new data presented herein suggest that, in concert with naturally-occurring Treg, hepatic pDC, that are comparatively abundant in the liver, may play a key role in regulation of alloimmune reactivity, with implications for the outcome of hepatic allografts and liver transplant tolerance.

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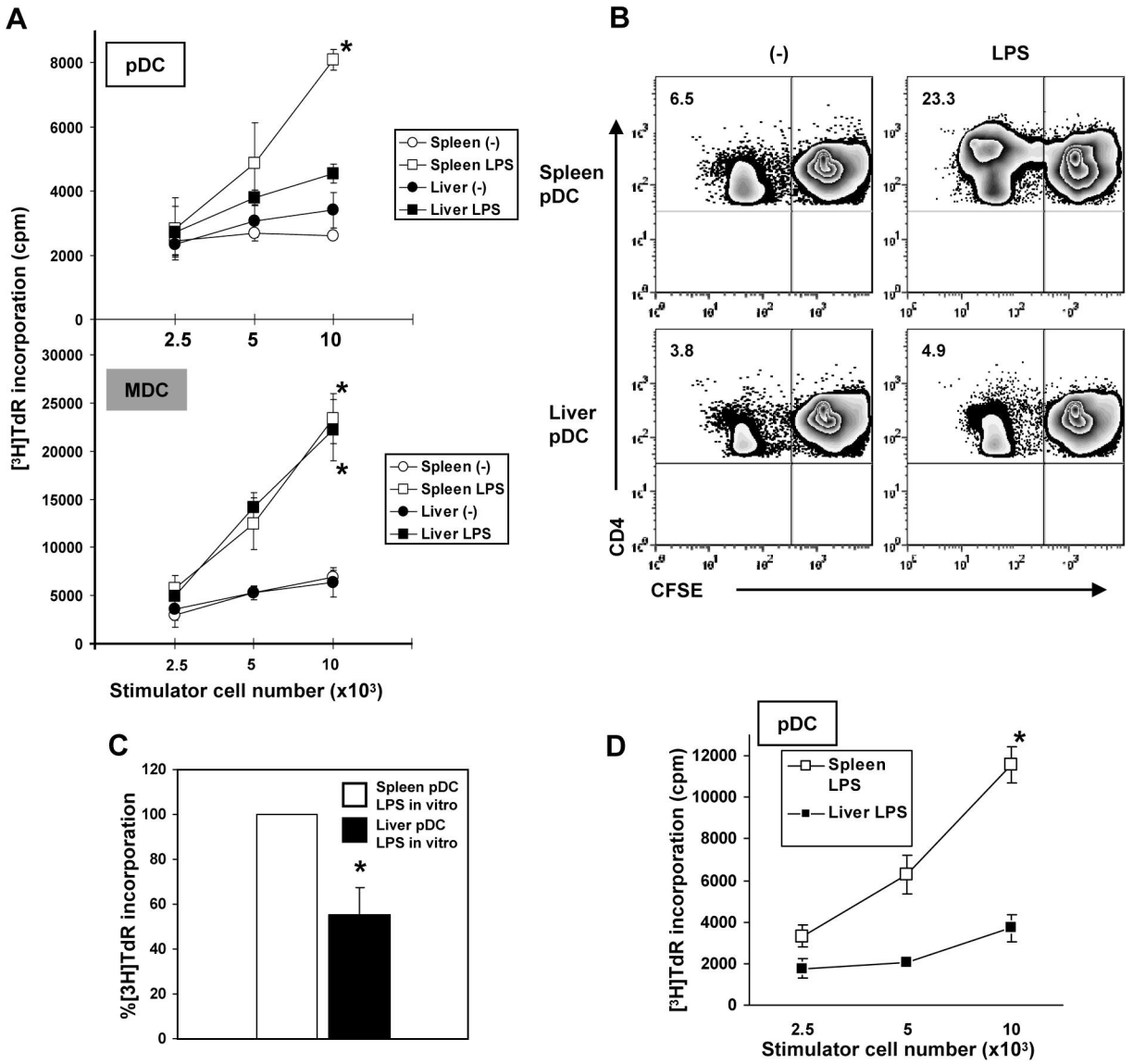

Fig. 1.

Liver pDC (immunobead-purified; mPDCA-1$^+$) from LPS-injected C57BL10 (B10) mice induce significantly lower proliferation of allogeneic CD4$^+$ T cells in 72h MLR, as determined by (A), [^{3}H]TdR incorporation, and (B), CFSE dilution analysis compared with spleen pDC (*p<0.01). The numbers in the upper left quadrants indicate percent positive CD4$^+$ T cells. A minimum of 20,000 CD4$^+$ gated cells were analyzed. In comparison, LPS-stimulated mDC from spleen or liver induced much higher T cell proliferation compared with untreated liver mDC (A; *p<0.01). (C), pDC from liver or spleen were cultured overnight (16h) with LPS (1μg/ml), then used as stimulators in MLR. Results are expressed as relative [^{3}H]TdR incorporation induced by spleen pDC as stimulators (*p<0.01). (D), Allostimulatory capacity of liver and spleen pDC from LPS-injected normal (non-Flt3L-mobilized) B10 mice (*, p<0.01). Data are representative of 5 (A and B) or 2 independent experiments (D), or means ± 1SD of 3 experiments (C).

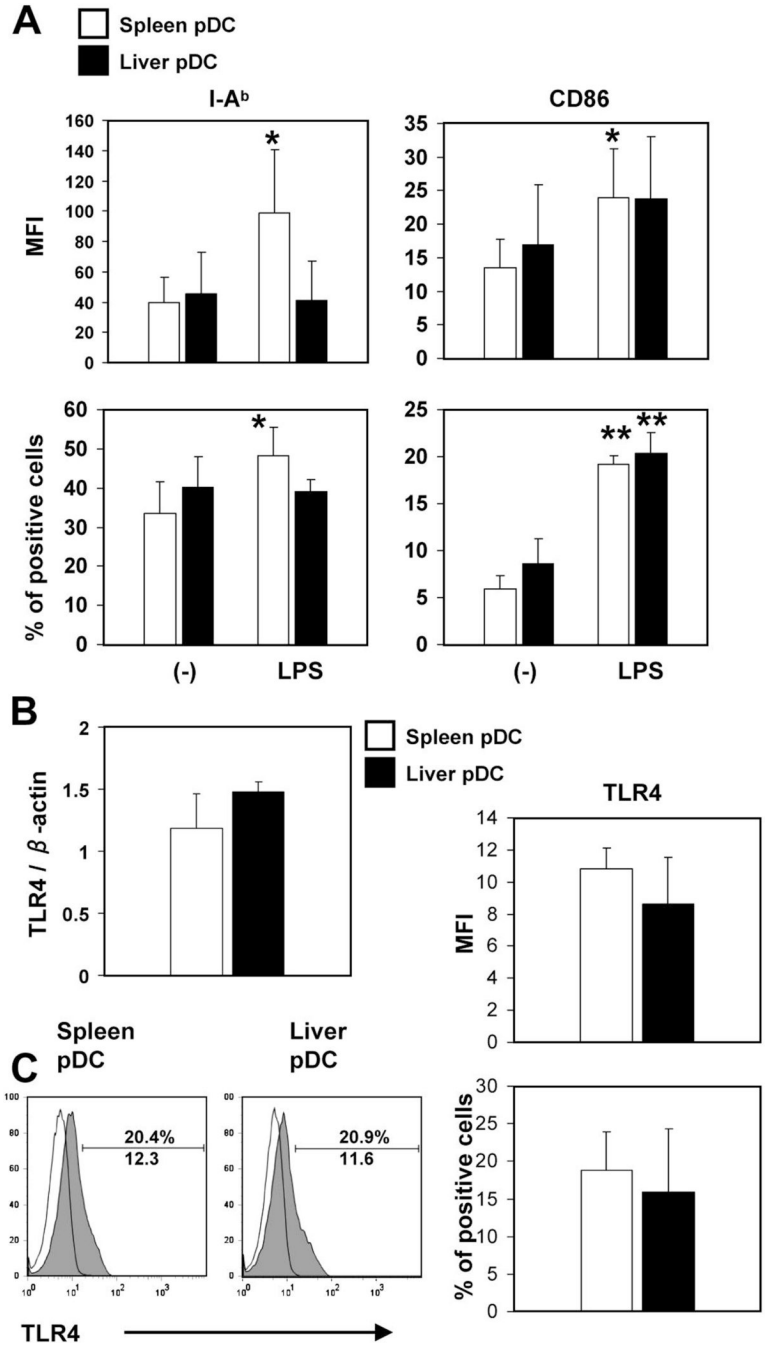

Fig. 2.

(A), Freshly-isolated liver and spleen pDC from control mice (no LPS administration) display similar surface levels of MHC class II (IA^b) and costimulatory (CD86) molecules. Following LPS administration, MHC class II and CD86 were upregulated on spleen pDC, but only CD86 (incidence of positive cells) was upregulated on liver pDC (*$p<0.05$, **$p<0.01$, compared with control mice) (B), The expression of TLR4 does not different significantly between freshly-isolated liver and spleen pDC from control mice, as determined by quantitative real-time RT-PCR. RNA was isolated from freshly-isolated, spleen or liver $mPDCA1^+$ cells and RT-PCR for TLR4 and β actin performed. Results are expressed as relative TLR4 mRNA expression normalized to β actin RNA. (C), Cell surface TLR4 staining confirmed similar levels on

freshly-isolated liver and spleen pDC. Staining shown is for the mPDCA1$^+$ pDC population. Open histograms represent appropriate Ig isotype control staining. A minimum of 20,000 CD11c$^+$ B220$^+$ gated cells were analyzed. Data are representative of 3 independent experiments (C), or means ± 1SD of 4 (A and B) or 3 experiments (C).

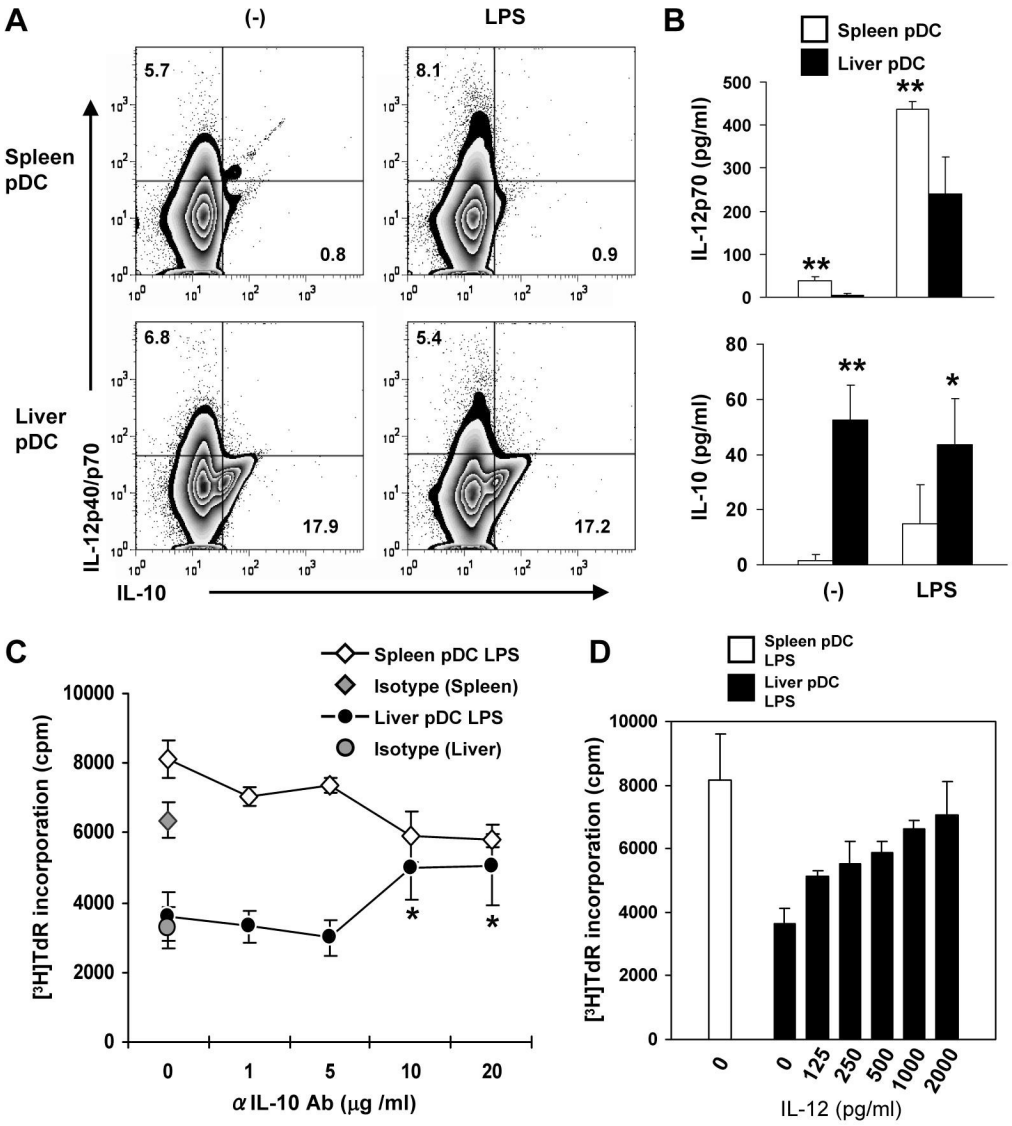

Fig. 3.

Unstimulated and LPS-stimulated liver pDC produce higher levels of IL-10 than splenic pDC. IL-12 and IL-10 were quantified by (A), intracellular staining, and (B), ELISA, as described in the Materials and Methods. Flow cytometric analysis of intracellular cytokine production and ELISA confirmed similar low levels of IL-12, but higher levels of IL-10 production by unstimulated and LPS-stimulated liver pDC compared with spleen pDC. Thus, the inferior T cell allostimulatory ability of LPS-stimulated liver pDC is associated with their differential regulation of pro-(IL-12) and anti-inflammatory (IL-10) cytokine production. The numbers indicate percent positive $CD11c^+$ $B220^+$ cells. A minimum of 20,000 $CD11c^+$ $B220^+$ gated cells were analyzed. (C), Addition of neutralizing anti-IL-10 mAb at the start of 72hr MLR cultures enhanced T cell proliferative responses induced by LPS-stimulated liver pDC, but not spleen pDC. *$p<0.05$ compared with untreated cultures, isotype control cultures, or mAb concs $\leq 5\mu g/ml$. (D), Addition of exogenous recombinant mouse IL-12 at the start of 72hr MLR cultures enhanced T cell proliferative responses induced by LPS-stimulated liver pDC. Data are representative of at least 3 (A and C) or 2 independent experiments (D), or means ± 1SD of 4 experiments (B). *, $p<0.05$; **, $p<0.01$.

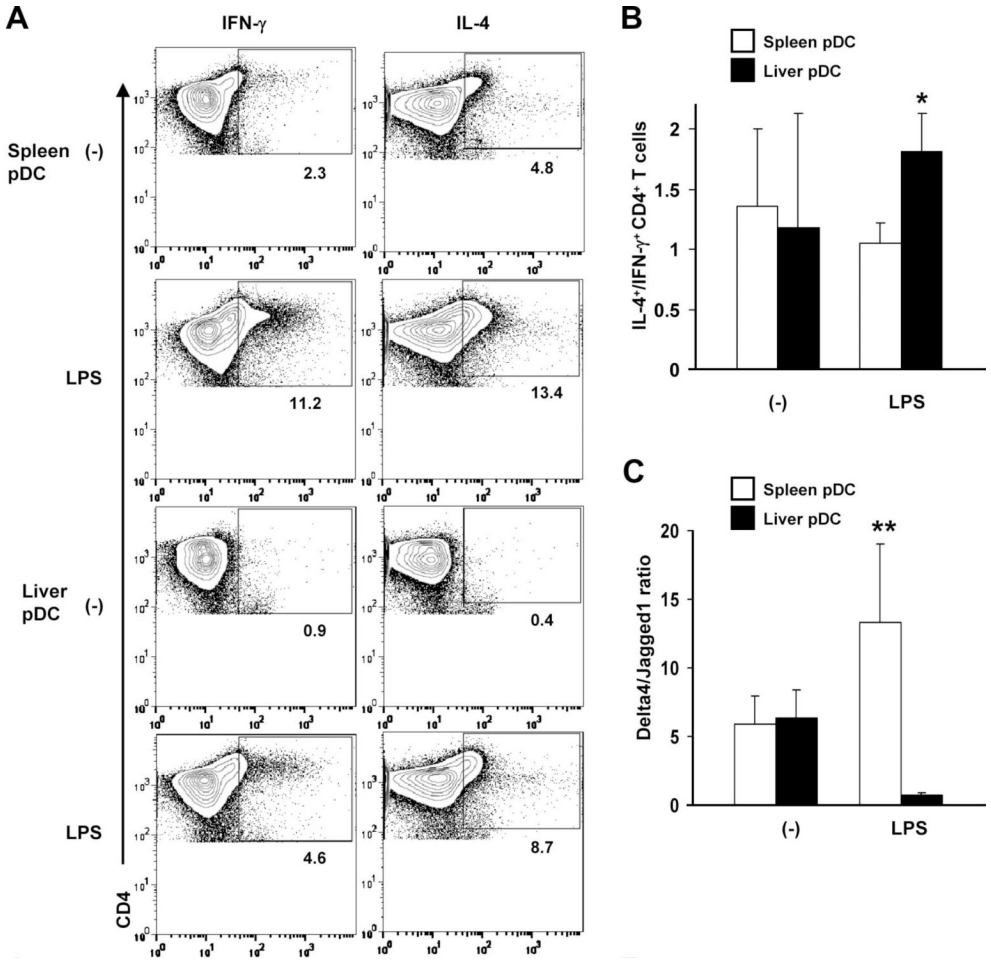

Fig. 4.

(A), The incidence of IFNγ- and IL-4-producing allogeneic $CD4^+$ T cells stimulated by LPS-activated liver pDC in MLR was lower than that induced by LPS-activated splenic pDC. The numbers indicate percent positive $CD4^+$ T cells. A minimum of 20,000 $CD4^+$ gated cells were analyzed. (B), Ratio of IL-4/IFNγ-producing $CD4^+$ T cells in 96 hr MLR cultures stimulated with LPS-stimulated liver pDC compared with spleen pDC. (C), The Delta4/ Jagged1 (Notch ligand) mRNA ratio expressed by freshly-isolated, LPS-activated liver pDC was much lower than that of activated spleen pDC. Data are representative of 3 experiments (A), or means ± 1SD of 3 (B) or 4 experiments (C). *, $p<0.05$; **, $p<0.01$.

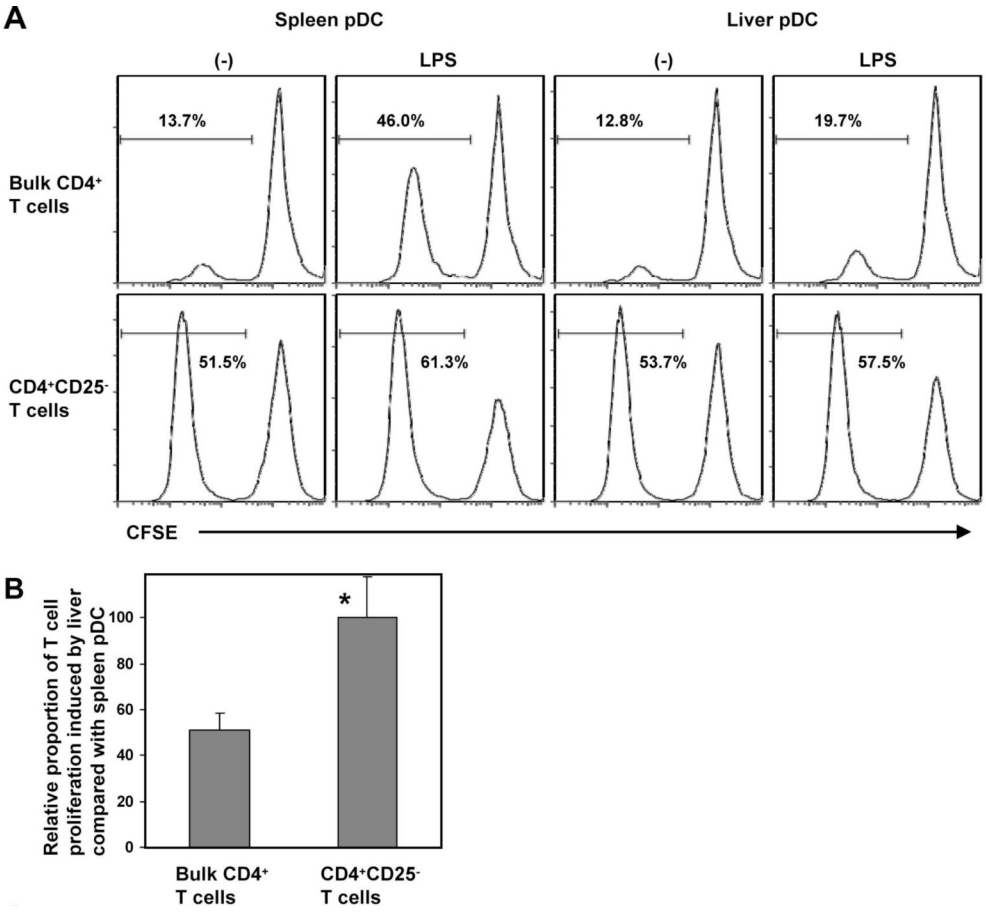

Fig. 5.

Naturally-occurring Treg ($CD4^+CD25^+$) contribute to the poor allostimulatory activity of liver pDC. (A), When $CD4^+CD25^+$ Treg were removed from the responder T cell population at the start of MLR, similar extents of CFSE-labeled T cell proliferation were observed when liver or spleen pDC were used as stimulators, reversing the inferior T cell allostimulatory activity of either unstimulated or LPS-stimulated liver pDC. The numbers indicate percent proliferating $CD4^+$ T cells. A minimum of 20,000 $CD4^+$ gated cells were analyzed. (B), confirms the statistical significance of the observed effect for pDC from LPS-treated mice. Data are representative of 3 independent experiments (A), or means ± 1SD of 3 experiments (B); *$p<0.05$.

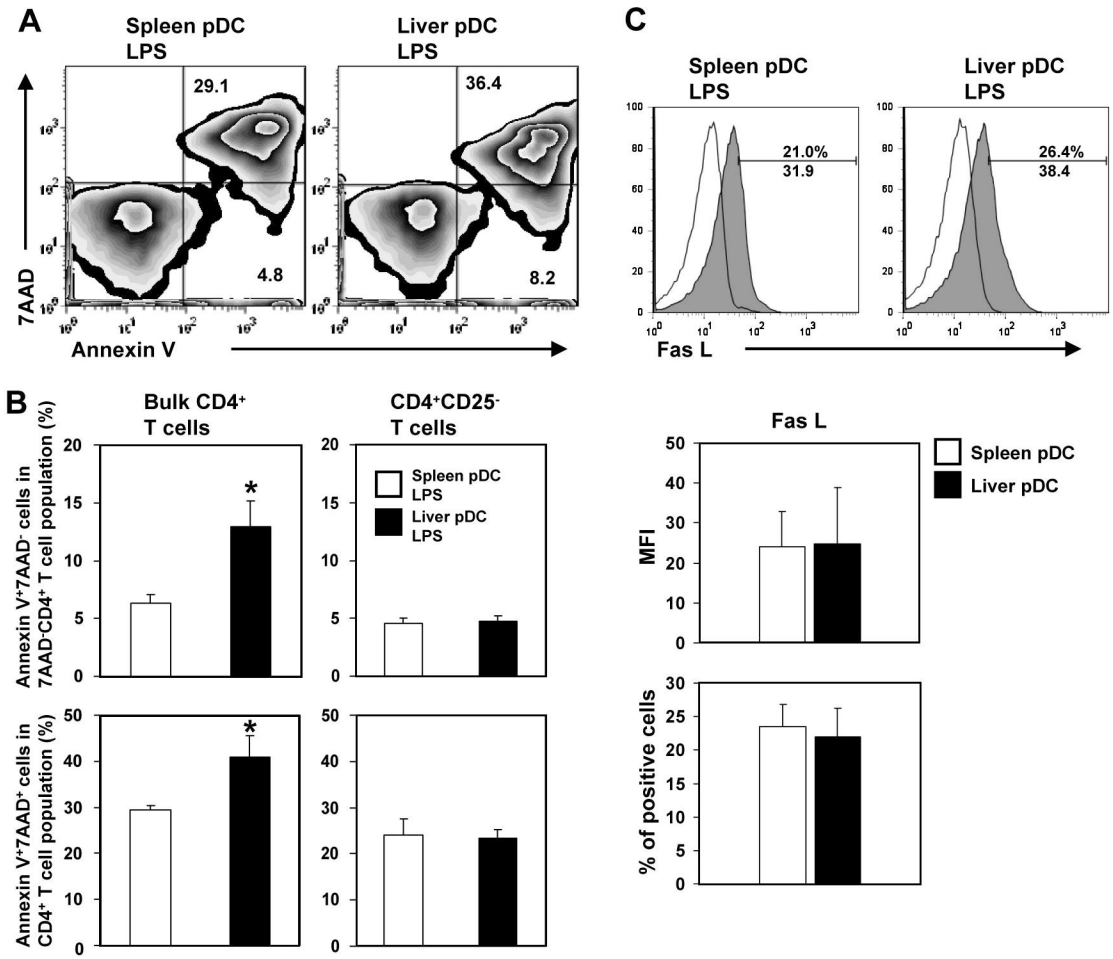

Fig. 6.

(A, and B - left side), LPS-activated liver pDC induce higher incidences of early (Annexin V^+ $7AAD^-$) and late apoptotic cells (Annexin V^+/7-AAD^+) in responder allogeneic $CD4^+$ T cell populations in 72h MLR compared with splenic pDC. The numbers indicate percent positive $CD4^+$ T cells. (B - right side), Removal of $CD4^+$ $CD25^+$ Treg immediately before the start of the MLR reversed the greater proapoptotic effect of liver pDC. (C), Freshly-isolated liver and spleen pDC from LPS-injected mice display similar surface levels of Fas Ligand (CD95L). Open histograms represent Ig isotype control staining. A minimum of 20,000 $CD4^+$ (A and B) or $CD11c^+$ (C) gated cells were analyzed. Data are representative of 3 independent experiments (A and C), or are means ± 1SD of 3 experiments (B and C);*P<0.05.