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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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***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-treated 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.**

***Conclusions*: 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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Abbreviations: 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

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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), $IA^b\beta$-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-