Induction of IDO

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Hepatic Stellate Cells Undermine the Allostimulatory Function of Liver Myeloid Dendritic Cells via STAT3-Dependent Induction of IDO

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Hepatic stellate cells (HSCs) are critical for hepatic wound repair and tissue remodeling. They also produce cytokines and chemokines that may contribute to the maintenance of hepatic immune homeostasis and the inherent tolerogenicity of the liver. The functional relationship between HSCs and the professional migratory APCs in the liver, that is, dendritic cells (DCs), has not been evaluated. In this article, we report that murine liver DCs colocalize with HSCs in vivo under normal, steady-state conditions, and cluster with HSCs in vitro. In vitro, HSCs secrete high levels of DC chemoattractants, such as MIP-1α and MCP-1, as well as cytokines that modulate DC activation, including TNF-α, IL-6, and IL-1β. Culture of HSCs with conventional liver myeloid (m) DCs resulted in increased IL-6 and IL-10 secretion compared with that of either cell population alone. Coculture also resulted in enhanced expression of costimulatory (CD80, CD86) and coinhibitory (B7-H1) molecules on mDCs. HSC-induced mDC maturation required cell–cell contact and could be blocked, in part, by neutralizing MIP-1α or MCP-1. HSC-induced mDC maturation was dependent on activation of STAT3 in mDCs and, in part, on HSC-secreted IL-6. Despite upregulation of costimulatory molecules, mDCs conditioned by HSCs demonstrated impaired ability to induce allogeneic T cell proliferation, which was independent of B7-H1, but dependent upon HSC-induced STAT3 activation and subsequent upregulation of IDO. In conclusion, by promoting IDO expression, HSCs may act as potent regulators of liver mDCs and function to maintain hepatic homeostasis and tolerogenicity. The Journal of Immunology, 2012, 189: 3848–3858.

Despite an elaborate in-house network of immune cells (NK cells, NKT cells, Kupffer cells [KCs], dendritic cells [DCs], and T cells) (1, 2), the liver exhibits remarkable tolerogenic properties, as evidenced by its retention of pathogens (e.g., the malaria parasite, and hepatitis B and C viruses), and its roles in oral and portal venous tolerance and tumor metastasis. This tolerogenic state is exemplified by the acceptance of liver allografts across MHC barriers without immunosuppressive therapy in animal models (3, 4), and the relative ease of acceptance of human liver transplants (5–8). However, acute or chronic liver graft rejection in a significant number of cases continues to be a major clinical challenge because of inadequate understanding of the mechanisms by which the hepatic immune system promotes and maintains tolerance.

Professional liver-resident APCs (DCs) constitute <1% of the nonparenchymal cell (NPC) population, yet they play an important role in regulation of ischemia–reperfusion injury (9, 10), liver transplant rejection (11), and hepatic fibrosis (12, 13). DCs acquire Ag in the local microenvironment and migrate to secondary lymphoid tissues, where they present processed Ag to T cells bearing Ag-specific receptors. DCs express costimulatory molecules—in particular, CD80 and CD86—and other coregulatory molecules, such as B7-homolog-1 (B7-H1; programmed death ligand-1), and induce Ag-specific T cell responses that mediate allograft rejection or acceptance (14).

Both myeloid (m) DCs and nonconventional plasmacytoid (p) DCs (15, 16) are found in the hepatic microenvironment (2). They display lower levels of costimulatory molecules and have poor T cell allostimulatory capacity compared with their counterparts in blood and secondary lymphoid tissues (17–20). Mechanistically, low cell surface expression of CD80 and CD86 by liver DCs is associated with IL-6–driven STAT3 activity (21, 22) in the steady state. STAT3 drives the induction of coinhibitory B7-H1 (23), as well as the immunoregulatory enzyme IDO (24). Notably, IDO-mediated control of T cell proliferation may play a role in hepatic tolerance (25–27). Although liver DCs have been shown to express IDO (28), the mechanisms underlying IDO induction in liver DCs are not well understood.
Hepatic stem cells (HSCs), localized in the perisinusoidal space, constitute 8–10% of total liver cells. They make direct contact with hepatocytes and with cells of the sinusoids, including sinusoidal endothelial cells and KCs, via their cytoplasmic extensions. In addition, HSCs display Ag-presenting and T cell costimulatory molecules, and produce various growth mediators and immune-modulating cytokines and chemokines (29–32). Thus, HSCs have potential to regulate the functions of many cell types, including hepatic immune cells. Indeed, HSCs can present lipid and peptide Ags to NKT and CD4+CD8+ T cells, respectively (33); induce T cell apoptosis via a B7-H1–dependent mechanism (34); and promote IL-2–dependent expansion of regulatory T cells (Tregs) (35). HSCs also enhance DC- and TGF-β–mediated expansion of Tregs, but block TGF-β–induced differentiation of Th17 cells (36). Activation of HSCs is associated with increased expression of B7-H4 that inhibits CD3/CD28-induced activation or proliferation of CD4+ T cells (37). Furthermore, HSCs inhibit splenic DC-induced proliferation of CD8+ T cells in a CD54-dependent manner (38), and induce apoptosis of conventional CD4+ T cells while expanding Tregs (39). However, whether HSCs affect the maturation and function of hepatic-resident DCs directly is unknown.

In this investigation, we observed direct association between HSCs and DCs in situ. In vitro coculture experiments revealed that HSCs recruit and activate syngeneic liver mDCs, as evidenced by increased expression of CD80, CD86, and B7-H1. HSC-induced maturation of mDCs was contact dependent and STAT3 mediated. Compared with control liver mDCs, mDCs conditioned by HSCs were inferior stimulators of allogeneic CD4+ and CD8+ T cell proliferation because of STAT3-dependent induction of IDO. These novel data reveal, to our knowledge, a previously undocumented role for HSCs in modulating the phenotype and function of liver mDCs, and provide insight into the mechanisms used by liver mDCs to undermine T cell responses.

**Materials and Methods**

**Animals**

All protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee, in accordance with National Institutes of Health guidelines. Male 8–12-wk-old C57BL/6 (B6), B6.129-Indotm1Alm/J (IDO−/−) or BALB/c mice (The Jackson Laboratory) were reared on a B6 background bred from pairs provided by the University of Pittsburgh; a generous gift from Dr. A. Jake Demetriz, Department of Pathology, and B7-H1−/− on a B6 background bred from pairs provided by Dr. Lieping Chen (The Johns Hopkins University, Baltimore, MD) were provided free access to standard laboratory chow and water.

**Isolation and culture of HSCs**

HSCs were isolated from B6 mice, as described (39, 40). Briefly, livers were digested with collagenase- and protease-containing HBSS. Cells were separated from the digested livers and suspended in HBSS, and the suspension was filtered through nylon mesh. The filtrate was centrifuged 3 or 4 times at 50 g for 1 min to remove hepatocytes and cell debris, and the supernatant was then centrifuged at 1400 g for 7 min. The pellet containing NPs was suspended in HBSS, and after purification via Nycodenz gradient centrifugation, HSCs were suspended (1 × 10^6 cells/ml) in DMEM containing 10% FBS, 10% horse serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded on gelatin (0.1% in PBS)-coated plates at a density of 0.5 × 10^5/cm², and 20 min later, loosely adherent HSCs were harvested and reseeded in new six-well plates. The medium was renewed after 3 h and then on alternate days. Cell purity, as determined by desmin and glial fibrillary acidic protein staining, was consistently >95%. The cells were used on day 7 of culture, when nearly 90% cells expressed α-smooth muscle actin (α-sma) (a marker of HSC activation), while retaining significant retinoid content (Supplemental Fig. 1).

**Isolation of liver DCs**

DCs were isolated from B6 [wild-type (WT) or IDO−/−] mice injected for 10 d with the endogenous DC poietin Flt3 ligand (Flt3L) (Amgen, Seattle, WA) (10 μg per mouse per day, i.p.), as described (17, 19). Following collagenase digestion of the liver, hepatocytes were removed from the cell suspension by low-speed centrifugation. NPs were then enriched by centrifugation on a 30% (w/v) Histodenz (Sigma-Aldrich, St. Louis, MO) gradient, and depleted of NK1.1+ cells, using anti-NK1.1 PE Ab, followed by incubation with anti–PE-labeled magnetic beads (Miltenyi Biotec, Auburn, CA). The pDCs were separated by positive selection, using magnetically labeled anti–PDCA-1 Ab (Miltenyi Biotec). The mDCs were selected from the NK1.1+ PDCA-1− elutant with magnetically labeled anti–CD11c (Miltenyi Biotec). The purity of pDCs and mDCs was consistently >90%.

**Imaging of DC–HSC interactions in situ and in vitro**

Livers were harvested after untreated or Flt3L-treated mice were stained using rabbit anti-desmin Ab (Abcam) and hamster anti-mouse CD11c Ab (BD Pharmingen, San Diego, CA). Goat anti-rabbit Alexa 488 (green) and goat anti-hamster Cy3 (red) Abs were used, along with Alexa 647 Phalloidin (Invitrogen, Carlsbad, CA). Nuclei were stained with Hoechst stain (Sigma-Aldrich). To examine in vitro interactions, 2.5 × 10^5 mDCs, labeled with CFSE (CellTracker Green; Invitrogen) per the manufacturer’s instructions, were added to chamber slides containing HSCs (1.25 × 10^6). After overnight incubation in 500 μl RPMI 1640, the cocultures were fixed with 2% paraformaldehyde in PBS for 1 h. The cells were then permeabilized and stained for polymerized actin (rhodamine phalloidin, red; Invitrogen) and DNA (Hoechst stain, blue; Sigma-Aldrich). Images were acquired using an inverted Fluoview 1000 confocal microscope (Olympus, Peabody, MA). Stacks through the cells were reconstructed to produce three-dimensional images.

**Flow cytometric analysis of DC phenotype**

Freshly isolated DCs or DCs harvested after overnight culture with or without HSCs (attached to the culture plate) were stained in cell staining buffer (CSB)–PBS containing 0.5% BSA. nonspecific binding was blocked with CSB–PBS containing 2% goat serum and anti-CD16/32 (BD Biosciences, San Jose, CA), then the cells were labeled with the following Ab panels for flow cytometric analysis: anti-CD11c Pacific Blue, anti-B220 PE/CY7, anti-CD80 APC, anti-CD86 PE/Cy5, anti-B7-H1 PE and anti-I-A^d FITC Abs or anti-CD11c Alexa Fluor700, anti-B220 PE Cy7, anti-CD86 PE/Cy5, anti-I-A^d PE, anti-CD80 APC, anti-CD54 FITC and rabbit anti-desmin with goat anti-rabbit Pacific Blue or anti-CD1c Pacific Blue, anti-CD80 APC, anti-B7-H1 PE/Cy5, anti-CD86 FITC, and anti-IL-6R PE (BD Biosciences). In some experiments, cells were stained with anti-CD54 FITC and a primary rabbit anti-mouse desmin with a secondary goat anti-rabbit Pacific Blue. For intracellular IDO staining, cells were fixed with 2% paraformaldehyde, permeabilized with CSB containing 0.1% saponin, and then stained with a primary rat anti-mouse IDO Ab (BioLegend), followed by a secondary goat anti-rat FITC (Cell Signaling Technology, Beverly, MA). In some experiments, intracellular IL-6 staining (PE-labeled anti–IL-6 Ab; BioLegend) was also performed. A minimum of 20,000 live events were acquired on either a BD LSR II or a BD Fortessa (BD Biosciences). Data were analyzed using FlowJo v7.5.5 (Treestar).

**STAT3 inhibition**

mDCs (1 × 10^5) were treated with STAT3 Inhibitor VII (250 nM) (Calbiochem, San Diego, CA) for 1–2 h, washed with PBS, and then cultured with IL-6−/− HSCs, B6.129 control HSCs, or B6 HSCs (5 × 10^5) overnight. mDCs were collected and analyzed for expression of surface markers or for IDO, or were used in CFSE-dilution assays.

**CFSE-dilution assays**

Bulk CD4+ and CD8+ T cells were isolated from BALB/c spleens by negative immunomagnetic selection. RBC-depleted splenocytes were labeled with purified rat anti–mouse I-A^d, -CD16/32, -B220, -Gr-1, and -Ter119 mAbs (all from eBioscience, San Diego, CA), then depleted using magnetic negatively conjugated anti-rat mAbs (Dynal Beads; Invitrogen, Auburn, CA). The pDCs were separated by positive selection, using magnetically labeled anti–CD11c (Invitrogen) per the manufacturer’s instructions and cocultured either with liver DCs harvested from liver DC/HSC cocultures or with DCs that had been cultured alone in parallel overnight. T cells (1 × 10^6) were stimulated with DCs (1 × 10^5) for 4 d, harvested, and then stained with anti-CD4 APC and anti-CD8 Pacific Blue (BD Biosciences). In some experiments, an IDO inhibitor, 1-methyltryptophan (1-MT; 200 μM; Sigma-Aldrich) or vehicle control (0.1N NaOH) was added at the start of the culture. A minimum of 20,000 live events were acquired on either a BD LSR II or a BD Fortessa. Data were analyzed using FlowJo v7.5.5.
Quantification of cytokines and chemokines

Cell-free supernatants were collected from overnight cultures of DCs (1 × 10^6), HSCs (5 × 10^4), or HSC+DCs in complete RPMI 1640 medium containing 10% FCS (Hyclone Laboratories, Logan, UT). Cytokines and chemokines in the supernatants were quantified using a Luminex 100 IS (BioLegend). Quantification of cytokines and chemokines in the supernatants was performed by ELISA (BioLegend).

Western blot analysis for suppressor of cytokine signaling 3

CD11c^+ liver mDCs recovered following overnight culture (without or with HSCs) were washed twice with ice-cold PBS and lysed in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 20 min. Proteins in the cell lysates (50 µg) were resolved by 12% PAGE, transferred onto polyvinylidene difluoride membrane, and blocked with 5% skim milk (in PBS containing 0.1% Tween 20) for 2 h. Total suppressor of cytokine signaling 3 (SOCS3) was probed using primary rabbit-polyclonal anti-SOCS3-A (a kind gift from Dr. Giorgio Raimondi, Thomas Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, PA) overnight at 4°C and donkey anti–rabbit-HRP Ab (GE Healthcare UK Limited, Buckinghamshire, UK), and detected using ECL Western Blotting Detection Reagent (GE Healthcare). Densitometric analysis was carried out using ImageJ software. Expression of total SOCS3 was normalized with β-actin as internal control.

Statistical analysis

Data were analyzed by the Student paired t test, using Excel (Microsoft). A value of p < 0.05 was considered significant.

Results

Liver DCs and HSCs colocalize under homeostatic conditions in vivo and in vitro

Liver DCs localize predominantly in the portal areas of normal liver tissue (41, 42). However, in addition to portal myofibroblasts (Fig. 1A), confocal imaging of normal B6 mouse liver identified CD11c^+ cells in proximity to HSCs (Fig. 1B). This spatial relationship clearly indicated close physical association between HSCs and DCs, and should be considered highly significant because DCs constitute <1% of the liver cell population and HSCs ~8–10%. HSC–DC interaction increased markedly following mobilization of DCs into the liver by treatment of mice with Flt3L (Fig. 1C, 1D). Overnight coculture of HSCs with freshly isolated syngeneic liver CD11c^+PDCA1^-NK1.1^- conventional mDCs resulted in accumulation of mDCs around HSCs (Fig. 1E, 1F), mimicking their in vivo interaction. These observations suggest that interactions between HSCs and DCs may have functional consequences.

HSCs express ICAM-1 (CD54) (38, 43) and desmin (29, 30) (Supplemental Fig. 2A). Liver mDCs cultured with HSCs upregulated CD54 expression but displayed low levels of desmin, verifying that the nonadherent CD11c^+ cells from HSC–DC cocultures were DCs, not HSCs (Supplemental Fig. 2A). Furthermore, mDCs were much smaller than HSCs (Figs. 1G, 1H, Supplemental Fig. 2B), providing an additional means to distinguish DCs from contaminating HSCs by flow cytometry.

Interactions between liver mDCs and HSCs modulate secretion of cytokines and chemokines

HSCs produce numerous chemokines and cytokines that may influence the immunologic activity of liver DCs. To assess whether interaction between these two cell types might influence each other’s cytokine and chemokine production in the hepatic microenvironment, supernatants were collected at 24 h from mDC or HSC mononuclears or HSC±mDC cocultures, and analyzed for specific cytokines and chemokines by Luminex. HSCs cultured alone secreted high levels of MCP-1, CXCL1, and MIP-1α (CCL3) (Fig. 2A), suggesting their possible involvement in recruitment of monocytes and neutrophils in the liver. HSCs may also play a direct role in innate immunity, because CXCL1 and MIP-1α possess antibacterial (44) and antiviral activity (45), respectively. Coculture of HSCs with mDCs did not alter the levels of MCP-1, CXCL1, and MIP-1α production (Fig. 2A). HSCs secreted IFN-γ-induced protein 10, which recruits lymphocytes, neutrophils, and monocytes, whereas mDCs secreted low levels of monokine induced by IFN-γ (CXCL9), which selectively recruits activated DCs; secretion of both of these chemokines increased markedly when mDCs and HSCs were cultured together. These data suggest that interaction between mDCs and HSCs may lead to enhanced recruitment of immune cells into the hepatic microenvironment and that HSCs may induce DC chemotaxis.

HSCs and mDCs, cultured alone overnight, produced the proinflammatory cytokines TNF-α and IL-1β, respectively, and both cell types produced low levels of IL-1α (Fig. 2B). Coculture of HSCs with mDCs did not alter the levels of TNF-α, IL-1β, or IL-1α significantly, compared with their levels as detected in cultures of either HSCs or mDCs alone. Neither cell type produced detectable levels of IFN-γ (data not shown). HSCs produced IL-10 (Fig. 2B), which is known to suppress DC maturation (46), and its production was unchanged when mDCs were cultured with HSCs (Fig. 2B). Secretion of IL-6, an important regulator of DC differentiation (47) and liver DC activation (21), was increased significantly in cocultures of liver mDCs and HSCs (Fig. 2B). Secretion of IL-6 by mDCs (Fig. 2), as well as its intracellular expression without or with HSC conditioning (Fig. 3A, lower panel), was relatively low. Thus, HSCs are the primary source of secreted IL-6 in the coculture. Cytokines typically associated with T cell differentiation and proliferation (i.e., IL-2, IL-4, IL-5, IL-12p70, IL-13, and IL-17) were not detected in supernatants from either mDCs or HSCs cultured alone or together (data not shown). These data indicate that interaction between HSCs and mDCs alters the cytokine milieu, with implications for the activation state or function of liver mDCs.

Liver mDCs upregulate costimulatory and coregulatory molecules when cultured with HSCs

Upregulation of CD54 on APCs correlates with their activation (48), and mDCs cocultured with HSCs upregulated CD54 (Sup-
Therefore, we evaluated expression of conventional activation markers, MHC class II (I-Ab), CD80, CD86, and B7-H1, on liver mDCs cultured with HSCs. As shown in Fig. 4A and 4B, coculture of mDCs with HSCs increased the incidence of mDCs expressing CD80, CD86, and B7-H1, as well as the level of expression of these molecules determined by mean fluorescence intensity (MFI) (Fig. 4C). These effects of HSCs on mDCs could be titrated by altering the HSC/mDC ratio in the cocultures (data not shown).

Because nonconventional pDCs account for ~20% of the total mouse liver DC population (49), the ability of HSCs to modulate the activation of liver pDCs was also tested. Liver pDCs cultured overnight with HSCs failed to upregulate cell surface CD80 and CD86 expression (data not shown). However, coculture of HSCs with splenic mDCs resulted in upregulation of DC maturation markers, similar to the effect of HSCs on liver mDCs (data not shown). Collectively, these data show that HSCs selectively modulate mDCs.

**HSC-induced expression of coregulatory molecules on liver mDCs requires cell–cell contact**

Because HSCs and DCs associate in vitro and in vivo (Fig. 1), and others have reported a need for cell–cell contact in order for DCs to activate HSCs (12), we examined the ability of HSCs to promote the phenotypic maturation of mDCs as a result of their physical association. When cell–cell contact was prevented using a Transwell system, the ability of HSCs to upregulate the expression of CD80, CD86, and B7-H1 on mDCs was markedly inhibited (Fig. 4A, 4D).

HSCs secrete MCP and MIP-1α (Fig. 2), which are potent DC chemoattractants (50). When cocultured in the presence of MCP- or MIP-1α-neutralizing Abs, the capacity of HSCs to promote CD80, CD86, and B7-H1 expression on mDCs was reduced, anti–MIP-1α being modestly, though not statistically, more effective than anti-MCP (Fig. 4E). The influence of these chemokines was not additive or synergistic, as indicated by no further change in the expression of coregulatory molecules when both anti-MCP and anti–MIP-1α were added to the cocultures. Collectively, these data suggest that the ability of HSCs to promote CD80, CD86, and B7-H1 expression on mDCs requires direct cell contact and that this contact may be mediated, in part, by HSC-secreted chemokines.

**Fas–FasL interactions do not play a significant role in the ability of HSCs to upregulate costimulatory molecules on liver mDCs**

Unlike other hematopoietic cells, DCs are resistant to Fas–FasL–induced apoptosis (51, 52). Indeed, Fas–FasL interactions can induce DC maturation (51, 53), increase IL-1β secretion (53), and inhibit DC apoptosis via NF-κB activation (53). Because HSCs express Fas (CD95) (39, 54), and direct contact is required for HSCs to promote mDC maturation (Fig. 4A, 4D), we hypothesized that Fas–FasL interaction might mediate the upregulation of CD80, CD86, and B7-H1 on mDCs cocultured with HSCs. Flow analysis of mDCs cultured overnight with HSCs revealed only modest upregulation of surface FasL (CD95L) (Supplemental Fig. 3A), and addition of neutralizing anti-CD95L mAb at the start of HSC–mDC cocultures caused no significant change in expression of coregulatory molecules (Supplemental Fig. 3B).

**HSC-derived IL-6 promotes liver mDC maturation**

Previous studies suggest that IL-6 impairs the differentiation of DCs (47), including liver DC maturation (21). In contrast, IL-6, in concert with IL-1β and TNF-α, can act as a potent activator of DCs (55). Because high levels of IL-6, TNF-α, and IL-1β were observed in HSC–mDC cocultures (Fig. 2B), we investigated the role of IL-6 as either an inducer or an inhibitor of HSC-mediated mDC maturation. To this end, we cocultured mDCs with HSCs isolated from either WT (B6.129) or IL-6−/− mice, and found that HSCs were the primary source of secreted IL-6 (Fig. 3A, upper panel). Intracellular staining confirmed that liver mDCs express very low levels of IL-6, which was not altered by coculture with HSCs (Fig. 3A, lower panel), and our previous work has demonstrated a high level of intracellular IL-6 expression by HSCs (39). Fewer mDCs conditioned with IL-6−/− HSCs upregulated CD80, CD86, and B7-H1 (Fig. 3B). IL-6−/− HSCs induced a lower level of expression of these markers, when compared with
that of WT HSCs (Fig. 3B, 3D). These data suggest that IL-6 plays a role in HSC-mediated maturation of liver mDCs.

Intracellular signaling coupled to DC activation involves transcriptional regulation via STAT1, STAT3, and NF-κB (56). IL-6 signals through STAT3, and STAT3 activation is reported to be critical for IL-6–driven B7-H1 upregulation on DCs exposed to low levels of TLR4 stimulation (23). To delineate transcriptional events that may be involved in HSC-induced mDC activation, mDCs were pretreated with a STAT3 inhibitor prior to culture with HSCs. STAT3 inhibition markedly reduced the expression of CD80, CD86, and B7-H1 on WT HSC-conditioned mDCs (Fig. 3C, 3D), suggesting a requirement for STAT3 activation in HSC-induced mDC maturation.

HSC-conditioned liver mDCs exhibit impaired T cell allostimulatory function in a STAT-3–dependent, but B7-H1–independent, manner

Activated DCs are known to induce activation or proliferation of CD4+ and CD8+ T cells. Because HSCs upregulated maturation markers (CD80, CD86, and B7-H1) on mDCs, we investigated the influence of HSC-conditioned mDCs on naive allogeneic T cell proliferation. Either liver mDCs or HSCs cultured alone were used as controls. HSCs alone failed to induce the proliferation of CD4+ or CD8+ T cells in CFSE-MLR, whereas liver mDCs induced the proliferation of both CD4+ and CD8+ T cells (Fig. 5A). Conditioning mDCs with HSCs, when compared with control mDCs, strongly inhibited their ability to induce allogeneic CD4+ and CD8+ T cell proliferation (Fig. 5A, 5C).

The expression of B7-H1, a potent coinhibitory molecule (57), was upregulated markedly on HSC-conditioned liver mDCs (Fig. 4A) and driven in part by HSC-derived IL-6 and STAT3 activation in mDCs (Fig. 3). We hypothesized that this mechanism might account for the diminished allostimulatory capacity of HSC-conditioned mDCs. To test this idea, mDCs conditioned by HSCs from WT or IL-6-/- mice were used as stimulators of naive allogeneic T cells. IL-6-/- HSC-conditioned mDCs were consistently better stimulators of CD8+ allogeneic T cells, but exhibited equivalent ability to stimulate CD4+ T cells compared with WT HSC-conditioned mDCs (data not shown). However, inhibition of STAT3 activation in liver mDCs prior to culture with HSCs restored their ability to stimulate both CD8+ and CD4+ T cell proliferation (Fig. 5B, 5C). Of interest, inhibition of STAT-3 in mDCs cultured alone increased their ability to induce T cell proliferation, which was enhanced when mDCs were conditioned by HSCs.

Given that HSCs cause pronounced upregulation of B7-H1 in liver mDCs, we postulated that conditioning B7-H1-/- mDCs with HSCs would enhance their ability to induce proliferation of T cells. However, B7-H1-/- liver mDCs, conditioned by HSCs, also exhibited reduced ability to stimulate proliferation of allogeneic T cells (Supplemental Fig. 4A), as seen with WT mDCs (Fig. 5A). Collectively, these data suggest that a distinct mechanism, driven by STAT3 activation in mDCs, is responsible for HSC-induced impaired ability of liver mDCs to stimulate T cell proliferation.
versus presence of the Ab.

DC + HSC cultures containing isotype control from four independent DC + HSC cultures containing the indicated Ab normalized to the MFI of for the indicated markers. Values shown are means + 1 SD of the MFI of MIP-1

DCs cultured with HSCs, with or without Transwells. (1 SD, and represent fold change in the expression of surface markers on a minimum of three experiments. (Isotype control; thin line, mDCs cultured alone; thick solid line, mDCs

tained by gating on CD11c+ cells in the live gate. Shaded histogram, when cultured with HSCs. (Fig. 6–dependent manner, are unable to induce robust proliferation of CD8+ T cells. This is an example of a regulatory mechanism in which DCs fail to activate T cells because they do not express the required costimulatory molecules (32), their influence on cells of the innate and adaptive immune systems has attracted increasing attention (33, 38, 66). Initially, it was reported that IFN–γ–stimulated mouse HSCs could induce apoptosis of activated allogeneic CD4+ and CD8+ T cells (Fig. 7A). Blocking IDO activity in HSC-conditioned mDCs restored their ability to induce proliferation of CD4+ T cells to the levels seen with control mDCs, and also enhanced the ability of HSC-conditioned mDCs to induce proliferation of CD8+ T cells (Fig. 7B). To further confirm that the suppressive effect of HSC-conditioned mDCs was indeed due to upregulated IDO, we used mDCs isolated from IDO−/− mice. The ability of HSC-conditioned mDCs to suppress T cell proliferation was not apparent in IDO−/− mDCs (Fig. 7A, 7B). In addition, the allostimulatory potential of HSC-conditioned mDCs was markedly increased in a CFSE-MLR with excess (500 μM) L-tryptophan (60) (data not shown). This increase in allostimulatory capacity mirrors the greater expression of costimulatory molecules (Fig. 4) seen on HSC-conditioned mDCs, which may be masked by induction of IDO.

A similar effect was seen when B7-H1−/− hepatic mDCs conditioned with HSCs were used to stimulate allogeneic T cell proliferation in the presence of 1-MT (Supplemental Fig. 4). These data emphasize the importance of IDO over B7-H1 in HSC-induced impaired ability of mDCs to induce T cell proliferation, and support our hypothesis that HSC-induced STAT3 activation increases induction of IDO in mDCs.

Discussion

Very few studies have evaluated the functional relationship between liver DCs and other liver-resident NPCs, despite their physical association in vivo, and the well-established importance of the hepatic environment in the maintenance of tolerance (1, 2). Interactions between liver macrophages (KCs) and DCs are the best defined, with KCs reported to capture circulating DCs from the blood, using an N-acetylgalactosamine-specific C-type lectin-like receptor (61, 62). Moreover, MIP-1α–secreting KCs recruit DCs to inflammatory sites (63), and KCs can initiate transcriptional changes that promote liver DC tolerogenicity by secreting high levels of IL-10 (64). Thus, the role of KCs in promoting tolerance may be prominent in conditions with intact endothelium. However, cold preservation- and reperfusion-induced injury to the liver disrupts the endothelial barrier (65). This damage, together with sluggish blood flow due to hepatic vasoconstriction, allows direct and protracted interactions between cells in the sinusoids (such as liver DCs) and perisinusoidal HSCs that also produce IL-10. From these pathophysiologic events, it is clear that HSCs, which are similar in number to KCs in hepatic NPC populations, may also contribute to hepatic immune tolerance.

Because HSCs make contact with almost all liver cell types via cytoplasmic extensions (30) and express immune regulatory molecules (32), their influence on cells of the innate and adaptive immune systems has attracted increasing attention (33, 38, 66). Initially, it was reported that IFN–γ–stimulated mouse HSCs could induce apoptosis of activated allogeneic CD4+ and CD8+ T cells (34). Subsequent studies have shown that HSCs can promote IL-2–dependent expansion of Tregs (35) and control bacterial infection by activating NK T cells via a lipid Ag-presenting mechanism (33). These studies portray HSCs as regulators of T cell activation within the liver. However, whether HSCs affect the immunobiology of the liver’s professional migratory APCs (DCs) has not been investigated.

STAT3 activation increases IDO expression in HSC-conditioned liver mDCs, which suppress the proliferation of allogeneic T cells

We have shown that liver mDCs, matured phenotypically in an IL-6–dependent manner, are unable to induce robust proliferation of T cells. STAT3 regulates IDO transcription (24). Of interest, in the absence of SOCS3, IL-6 can activate DCs while causing simultaneous upregulation of IDO activity, and thus induces an activated, yet tolerogenic DC phenotype (58, 59). We hypothesized that the apparent discordant effects of HSC conditioning on liver mDC phenotype and function might result from increases in STAT3-driven IDO activity. In support of this, expression of IDO, as determined by flow cytometry, was significantly (1.7-fold) higher in mDCs conditioned by HSCs (Fig. 6A, 6B). The HSC-induced increase in IDO was contact dependent (data not shown) and blocked in mDCs treated with a STAT3 inhibitor prior to coculture with HSCs (Fig. 6A, 6B).

SOCS3 has been shown to regulate ubiquitination-mediated proteosomal degradation of IDO in DCs (59). We found that expression of total SOCS3 in mDCs was significantly downregulated by HSCs (Fig. 6C, 6D), suggesting that the increased IDO expression in mDCs is upregulated via HSCs by inhibiting SOCS3. To determine if the reduced T cell allostimulatory function of HSC-conditioned mDCs was due to increased IDO activity, an IDO inhibitor (1-MT) was added to CFSE-dilution assays, together with either control or HSC-conditioned mDCs. IDO blockade enhanced the ability of control hepatic mDCs to stimulate proliferation of CD4+ and CD8+ allogeneic T cells (Fig. 7A).

Blocking IDO activity in HSC-conditioned mDCs restored their ability to induce proliferation of CD4+ T cells to the levels seen with control mDCs, and also enhanced the ability of HSC-conditioned mDCs to induce proliferation of CD8+ T cells (Fig. 7B). To further confirm that the suppressive effect of HSC-conditioned mDCs was indeed due to upregulated IDO, we used mDCs isolated from IDO−/− mice. The ability of HSC-conditioned mDCs to suppress T cell proliferation was not apparent in IDO−/− mDCs (Fig. 7A, 7B). In addition, the allostimulatory potential of HSC-conditioned mDCs was markedly increased in a CFSE-MLR with excess (500 μM) L-tryptophan (60) (data not shown). This increase in allostimulatory capacity mirrors the greater expression of costimulatory molecules (Fig. 4) seen on HSC-conditioned mDCs, which may be masked by induction of IDO.

FIGURE 4. CD11c+ liver mDCs upregulate coregulatory molecules when cultured with HSCs. (A) Purified mDCs (1 × 106) were cultured overnight with HSCs (5 × 106), harvested, and stained with anti-CD11c and for the indicated DC maturation markers. Flow data shown were obtained by gating on CD11c+ cells in the live gate. Shaded histogram, Isotype control; thin line, mDCs cultured alone; thick solid line, mDCs cocultured with HSCs in contact; thick dotted line, mDCs conditioned with HSCs without contact. Data shown are representative of at least three independent experiments. (B) Percent positive cells compared with isotype controls for the indicated markers. Bars depict the means ± 1 SD from a minimum of three experiments. (C) The fold change in MFI for HSC-conditioned DCs relative to control DCs is depicted. Dotted line at a value of 1.0 represents DCs cultured alone. *p < 0.05. (D) Bars indicate mean ± 1 SD, and represent fold change in the expression of surface markers on DCs cultured with HSCs, with or without Transwells. (E) DCs were cultured with HSCs overnight in the absence or presence of anti-MCP or anti-MIP-1α neutralizing mAb (5.0 μg/ml). DCs were collected and stained for the indicated markers. Values shown are means ± 1 SD of the MFI of DC + HSC cultures containing the indicated Ab normalized to the MFI of DC + HSC cultures containing isotype control from four independent experiments. *p < 0.05, MFI from HSC-conditioned DCs in the absence versus presence of the Ab.
In this report, we show for the first time, to our knowledge, that HSCs interact physically with conventional liver mDCs, enhancing their expression of cell surface coregulatory molecules (CD80, CD86, and B7-H1). These effects are cell–cell contact dependent and require HSC-derived soluble mediators (MCP-1, MIP-1α, and IL-6). Other factors secreted by HSCs, including endothelin-1 and PGs (67–69), have been reported to modulate DC activation (70–72), but in the current study, pharmacologic inhibition of endothelin receptors did not affect HSC-induced liver mDC maturation (data not shown). Furthermore, addition of indomethacin only modestly reduced CD80 and CD86 expression on mDCs cultured with HSCs (data not shown). HSCs also express high levels of Fas (54) and induce modest expression of FasL on mDCs, but neutralizing FasL in mDC-HSC cultures had only a minor effect on maturation marker expression by liver mDCs. Likely, the upregulation of these markers on liver mDCs is due, at least in part, to the combined effects of multiple proinflammatory cytokines (TNF-α, IL-6, and IL-1β) secreted by HSCs in coculture with mDCs (Fig. 2).

We found that reducing the levels of IL-6 available to liver mDCs inhibited their expression of CD80, CD86, and B7-H1. Several groups have shown that liver DCs have comparatively poor T cell allostimulatory capacity compared to those cocultured with HSCs. These effects are cell–cell contact dependent and require HSC-derived soluble mediators (MCP-1, MIP-1α, and IL-6). We suggest that the impaired T cell allostimulatory function conferred by HSCs might be due to marked HSC-induced upregulation of B7-H1 on liver mDCs. However, our data show that the impaired T cell stimulatory activity of HSC-conditioned liver mDCs was independent of their B7-H1 expression.

Of interest, tolerogenic or regulatory DCs may express high levels of CD80, CD86, and B7-H1, yet also produce IDO, a potent enzyme that suppresses T cell proliferation (74). Altering the intracellular adaptor protein SOCS3 can change the signaling pattern of DCs, turning anti-inflammatory IL-6 into a DC-activating cytokine, but also inducing IDO and imparting a tolerogenic phenotype on the cells (58, 59). Recently, we have identified a role for DNAX-activating protein of 12 kDa (DAP12), a molecule associated with IDO induction, in the regulation of liver mDC maturation (19). Interactions with HSCs may induce changes in these intracellular molecules (SOCS3 and DAP12) and alter IL-6 signaling to induce a tolerogenic DC phenotype. Although our results are consistent with the reported positive association be-

**FIGURE 5.** CD11c+ liver mDCs cultured with HSCs have impaired T cell allostimulatory function. Purified liver mDCs (1 × 10^6) were cultured with HSCs (5 × 10^5) overnight, aspirated, washed, and then cultured with bulk CFSE-labeled allogeneic splenic (BALB/c) T cells (10^5 T cells:10^4 stimulators). (A) After 4 d of culture, T cells were collected and stained with anti-CD4 and anti-CD8. Data shown are representative of 10 experiments. The proliferation of T cells alone cultured in parallel is shown as the shaded histogram (overlaid). Numbers indicate the mean percentage of dividing cells ± SD from 10 experiments. (B) Purified mDCs were treated with vehicle or STAT3 Inhibitor VII (250 nM) before overnight culture with HSCs. HSC-conditioned or control mDCs were used to stimulate T cells as in (A), and T cell (CD4+ and CD8+) proliferation was measured by dilution in CFSE fluorescence. (C) Graphical representation of data shown in (B) is from 3 to 10 independent experiments (mean ± 1 SD) with consistent results. *p < 0.05, **p < 0.001.
tween serum IL-6 concentration and IDO expression in a murine model of sepsis (75), exogenous IL-6 failed to upregulate IDO expression in liver mDCs (results not shown). Furthermore, IL-10 also did not alter IDO expression in mDCs. We also considered a role for TGF-β, which is expressed at low levels in rat (76) and mouse HSCs (A. Dangi and C.R. Gandhi, unpublished observations), in the IDO increase in mDCs. However, incubation in the presence of anti–TGF-β Ab did not alter HSC-induced IDO expression in DCs (results not shown). These results indicate an alternate mechanism of HSC-induced IDO upregulation in DCs.

Nevertheless, in view of the significant role of IDO induction in immune regulation in experimental hepatitis (77, 78), HSC-induced IDO in liver mDCs may be important in regulating alloimmunity.

IL-6, which is produced by HSCs and further upregulated in HSC–mDC coculture, is known to signal via STAT3 activation (21, 56), an important transcriptional pathway for maintaining DC immaturity, particularly in the liver (21, 22). However, we found that blocking STAT3 activation in liver mDCs reduced their CD80, CD86, and B7-H1 expression, suggesting that, in some circum-

![FIGURE 6](image)

**FIGURE 6.** HSCs induce IDO expression in a STAT3-dependent manner in CD11c⁺ liver mDCs, resulting in inhibition of cell proliferation. (A) Purified mDCs were treated with vehicle control or STAT3 Inhibitor VII (250 nM), then cocultured with HSCs overnight, collected, and stained for intracellular IDO for flow cytometric analysis. (B) Bar graph shows the mean ± 1 SD of fold change in the MFI of IDO expression (normalized over the MFI values of isotype control). The data shown in (A) and (B) are from at least 3 to 10 independent experiments. (C) Total SOCS3 expression as determined by Western blotting analysis. (D) Bar graph shows densitometric readings for SOCS3 expression versus that of β-actin (n = 3, *p < 0.05, **p < 0.001).

![FIGURE 7](image)

**FIGURE 7.** Allostimulatory function of CD11c⁺ liver mDCs (WT or IDO⁻/⁻). (A) CFSE-labeled bulk splenic T cells were cultured with control or HSC-conditioned WT mDCs in the absence or presence of 1-MT (200 μM) or with HSC-conditioned IDO⁻/⁻ mDCs. After 4 d, CD4⁺ and CD8⁺ T cell proliferation was measured using flow cytometry (representative of 3–10 independent experiments with consistent results). (B) Graphical presentation (mean ± 1 SD of percent CD4⁺ and CD8⁺ T cell proliferation) of the data shown in (A). *p < 0.05, **p < 0.001.
stances, STAT3 can promote DC maturation. In support of this idea, we found previously that the majority of active STAT3 was expressed in CD80+ or CD86+ liver DCs (19). Earlier studies have shown that, in a tumor-specific environment, myeloid APCs mature in a contact-dependent manner via STAT3 activation (79, 80). Of interest, STAT3 activation was also associated with increased IDO expression in HSC-conditioned liver DCs. IDO induction may occur directly, through STAT3 acetylation (24), or may be induced indirectly, by shifting NF-κB signaling to a non-canonical pathway (81). Alternatively, STAT3 activation in mDCs may increase the expression of chemokine receptors involved in recruitment of DCs to HSCs, as has been reported for T cells (82).

In conclusion, our results further implicate HSCs as a cell type with a significant role in the development of a tolerogenic liver environment.

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Disclosures
The authors have no financial conflicts of interest.

References

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Supplemental Fig 1. HSCs express α-SMA and retain retinoids. HSCs on day 2 or 7 of culture were stained for desmin (green; a marker for HSCs) and α-SMA (red; a marker for activated HSCs) (A) and by Oil Red for intracellular retinoids (B). Negligible α-sma expression and abundant retinoids can be seen in HSCs on day 2. On day 7, α-SMA expression is increased markedly, but the cells still retain significant amounts of retinoids indicating the transiently-activated phenotype seen during early injury such as in transplantation.
Supplemental Fig 2. HSCs and liver mDCs differ in size, granularity and desmin content. Liver mDCs (CD11c<sup>+</sup>PDCA-1<sup>+</sup>NK1.1) or HSCs were cultured alone or together (1:20 ratio), harvested then evaluated by flow cytometric analysis. In (A), cells were stained for surface CD11c and CD54, and intracellular desmin. Data are shown for DCs gated on CD11c<sup>+</sup> cells. In (B), the size (forward scatter, FSC) and granularity (side scatter, SSC) are compared. The size of HSCs is so large that it cannot be captured using the FSC/SSC utilized to analyze DCs. Representative of 2 independent experiments.
Supplemental Fig 3. HSC-mediated activation of liver mDCs is independent of Fas/FasL interactions (A) Expression of Fas and FasL on liver mDCs cultured with or without HSCs (1x10⁶ DCs: 5x10⁴ HSCs) for 24h. (B) The expression of I-A^b, CD80, CD86 or B7-H1 on CD11c^+ cells cultured with either isotype control hamster Ig (broken line) or hamster anti-mouse FasL (5 μg/ml, solid line). Gray histograms show isotype control. Bar graph depicts relative expression of the markers indicated on CD11c^+ cells cultured with HSCs in the presence of anti-mouse FasL, normalized to the expression of the indicated markers expressed on CD11c^+ cells cultured with HSCs in the presence of isotype control (means ± 1SD from 3 independent experiments).
Supplemental Fig 4. HSC-conditioned B7-H1-liver mDCs suppress the proliferation of CD8+ and CD4+ T cells in an IDO-dependent fashion. (A) Liver mDCs were purified from B7-H1-liver mice cultured overnight with control B6 HSCs, harvested and used to stimulate CFSE-labeled bulk splenic T cells for 4d at a ratio of 10 T cells:1 DC. Data shown are gated on CD4+ or CD8+ T cells. Shaded histogram represents CFSE-labeled T cells cultured without DCs. In (A), numbers represent the mean ± SD of the dividing cells from 3 experiments. *p<0.05 comparing dividing T cells when stimulated with control or HSC-conditioned B7-H1-liver mDCs. (B) CFSE-dilution assays were performed as in (A) using control or HSC-conditioned B7-H1-liver mDCs to stimulated T cells in the presence or absence of 1-MT.