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Dendritic cell immunobiology in relation to liver transplant outcome

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1. ABSTRACT

The unique immunologic environment of the liver, together with its anatomic location downstream of the gut, influences the maturation and function of its interstitial dendritic cell (DC) populations. These well-equipped, antigen-presenting cells play critical roles in regulation of innate and adaptive immunity. New information is emerging about the molecular regulation of liver DC maturation and function, and their tolerogenic potential, while new insight is being gained regarding interactions between liver DC and other immune effector cell populations (NK, NKT cells) in addition to T cells. During transplantation, factors that affect liver DC biology include ischemia-reperfusion injury, liver regeneration, viral infection and the actions of anti-inflammatory and immunosuppressive drugs. Herein, we review the molecular and cell biology of hepatic DC populations in relation to the regulation of alloimmune responses and liver transplant outcome.

2. INTRODUCTION

Resident antigen (Ag)-presenting cells (APC) within the liver include leukocytes,-Kupffer cells (KC) and dendritic cells (DC), parenchymal cells (hepatocytes), sinusoid-lining endothelial cells and stellate (Ito) cells. These APC appear to have dual roles in initiating and regulating immune responses. DC play a key role in innate immunity and also orchestrate adaptive immune responses, integrating signals from the extracellular environment with Ag processing and presentation. The outcome of these interactions may be either immune stimulation or tolerance. As discussed herein, the unique liver microenvironment, and various factors related to liver transplantation, that include ischemia-reperfusion injury (IRI), liver regeneration, immunosuppressive drug administration, and viral infection, may determine how these cells influence alloimmune responses and graft outcome.

3. IMMUNOBIOLOGY OF LIVER DC

3.1. Phenotypic characteristics of DC

Various cell surface markers are used to identify and purify rodent and human DC (1-11). CD11c is commonly employed to identify DC in mice. Other Ags, e.g. the mannose receptor, CD205 (formerly DEC205), can help identify mouse DC. It is expressed at higher levels on mouse liver DC compared with spleen DC (12). OX62, an integrin molecule, is often used to identify rat DC. Human DC are lineage$^-$ HLA-DR$^+$. DC-SIGN (DC-specific intercellular adhesion molecule [ICAM]-3 grabbing non-integrin), a c-type lectin receptor, is used as a marker for immature DC. Recently, the human DC subset-specific markers, blood DC Ag (BDCA)-1 (CD1c), -2 (CD303), -3 (CD141) and -4 (CD304), have been identified and monoclonal antibodies directed against these Ags used to characterize these cells in blood and tissues.

At least four phenotypically and functionally distinct DC subsets have been identified in mouse secondary lymphoid tissue and the liver: conventional myeloid (m) DC (CD11c$^+$CD8alpha$^-$CD11b$^+$ or CD11c$^+$ PDCA-1$^-$), plasmacytoid pDC (CD11c^{lo}B220$^+$Ly-6C$^+$CD11b$^-$ or PDCA-1$^+$), CD8alpha$^+$ DC (CD11c$^+$CD8alpha$^+$CD11b$^-$) and natural killer (NK) DC (NK1.1$^+$ CD11c$^+$). mDC acquire Ag in the periphery, then migrate to lymph nodes to initiate T helper (Th) 1, Th2, Th17 or regulatory T cell (Treg) responses. pDC produce type-I interferon (IFN) in response to viral stimulation (13), and may influence the development or the strength of a Treg response (14). CD8alpha$^+$ DC have been shown to polarize naïve T cells towards Th1 responses (15), but also to promote organ allograft survival (16). NKDC present Ag to T cells. They also have lytic function (17) and produce IFNgamma via autocrine IL-12 in response to cytidylyl phosphate guanosine (CpG) stimulation (18). The relative abundance of these individual DC subsets differs between the mouse liver and spleen, with the liver containing higher incidences more pDC and NKDC and fewer mDC (3).

In humans, CD8alpha DC and NK DC have not been identified. While mDC (CD11c$^+$CD11b$^+$lineage [lin]$^-$ BDCA1$^+$ or CD11c$^+$CD123$^-$lin$^-$HLA-DR$^+$) and pDC (BDCA-2$^+$, BDCA-4$^+$ or lin$^-$ HLA-DR$^+$ CD11c$^-$ CD123^{hi} or CD4$^+$CD11c$^-$) have been described in humans, few studies have evaluated these subsets in human liver. mDC have been identified within human liver biopsies and liver perfusate (6). pDC have been identified in human hepatic lymph nodes (10). More recently, both mDC and pDC have been identified in liver tissue from hepatitis C virus (HCV)-infected and non-infected liver disease patients (11). Unfortunately, this study failed to characterize liver DC subsets from non-diseased controls.

3.2. DC migration in and out of the liver

DC are derived from CD34$^+$ hematopoietic stem cells and migrate to the liver from the blood via the hepatic sinusoids (19). Under steady-state conditions, small populations of DC are found within the perivenular region, portal space, and beneath the Glisson capsule, with a few DC scattered throughout the parenchyma (20, 21). Freshly-isolated immature murine liver DC express mRNA for CCR1, 2 and 5 (8). Rat DC precursors expressing CCR1 and CCR5 are recruited to the liver in response to macrophage inflammatory protein-1alpha secreted by KC during inflammation (22, 23). DC recruitment into the rat liver is further facilitated by the binding of KC to N-acetylgalactosamine on the DC (24).

DC differentiate while migrating from the portal to the central vein. These DC then cross the sinusoidal lumen through endothelial pores to the hepatic lymph via the space of Disse (25). In the space of Disse, and the portal tracts, DC form close contacts with lymphocytes (26). Under inflammatory conditions, they are recruited to portal tract-associated lymphoid tissue (PALT) in response to secondary lymphoid organ chemokine (SLC) (22). Within the PALT, DC activate T cells (22). Liver DC also prime T cells in the celiac lymph nodes following uptake of exogenous Ag and migration via the lymph. DC migrate from normal rat livers at a rate of approximately 10^5 DC/hr (27).

In humans, HCV proteins enhance the secretion of RANTES (the ligand for CCR5) and decrease the secretion of CCL21 (the ligand for CCR7) (28), which is involved in DC homing to secondary lymphoid tissue (22). HCV-DC interactions such as this may alter DC trafficking, favoring retention of immature and mature DC in the liver, and inhibiting subsequent DC-T cell interactions in the peripheral lymphoid tissues.

Following the severing of hepatic lymph vessels, as occurs during liver transplantation, liver-derived DC are found in the spleen and celiac lymph nodes (29). In a model of rat spontaneous liver allograft acceptance (PVG→DA), donor-derived DC can be identified in recipient celiac lymph nodes, shortly after transplant (30). Lymphocytes in these nodes express elevated IL-2 mRNA and display increased CD25 compared to those in nodes draining skin grafts (30). In the secondary lymphoid organs, donor leukocyte migration is associated with increased T cell apoptosis in animals receiving spontaneously accepted liver grafts compared with more readily rejected kidney grafts (31). These data suggest that donor DC may suppress T cell responses by the induction of recipient CD25$^+$ regulatory T cells (Treg) or activation-induced T cell death.

A greater number of donor MHC class II$^+$ cells are found in the host's spleen following rat liver transplantation compared to the number of MHC class II$^+$ cells in the spleen following transplantation of more readily rejected heart allografts (32). In rats tolerized by donor-specific transfusion (DST) prior to liver transplantation, donor hepatic DC (OX-62$^+$) migrate to the splenic red pulp whereas donor DC in control rats (no DST) are found only in the splenic white pulp (33). These findings are particularly interesting given the functions of the splenic red and white pulp, with red pulp involved in filtering of the blood, while the white pulp is thought to be the site of T cell activation (34). Retention of DC in the red pulp may prevent T cell activation in this model.

3.3. DC-T cell interactions

DC resident in normal peripheral tissue are immature, expressing low levels of MHC class II and co-stimulatory molecules. These immature DC scan the periphery for Ag and are well-equipped for Ag capture, processing and loading onto MHC class II molecules. Endogenous and exogenous 'danger signals', including tumor necrosis factor (TNF) alpha and pathogen-associated molecular pattern (PAMP) molecules, especially Toll-like receptor (TLR) ligands, induce DC maturation. Upon maturation, DC up-regulate expression of MHC class II, intercellular adhesion and co-stimulatory molecules (e.g. CD80, CD86, B7-H1 and inducible costimulatory ligand [ICOSL]) and synthesize large amounts of bioactive IL-12p70 or IL-4. Mature DC traffic to T cell areas of secondary lymphoid tissues in response to CCL19 or CCL21 gradients following up-regulation of CCR7. Interaction between DC expressing Ag in the context of MHC class II and the T cell receptor (TCR) on $CD4^+$ T cells leads to proliferation of Ag-specific T helper (Th) cells and differentiation into Th type-1 (Th1) or Th2 cells. DC also play a prominent role in inducing IL-17-producing Th cells (Th17) (35) and Treg (36).

Granulocyte/macrophage colony-stimulating factor (GM-CSF) has been used to expand liver DC progenitors *in vitro* (35, 37). These liver-derived mDC progenitors are functionally immature and weak stimulators of naïve allogeneic T cells (38), but also induce Ag-specific memory T cell proliferation (39). Liver-derived mDC progenitors have high surface expression of CD45, CD11b, CD24 and CD44, and moderate expression of CD11c and CD205 (37, 39). Injection of liver-derived mDC progenitors into allogeneic recipients elicits IL-10- and IL-4-producing T cells (40). By contrast, transfer of mature liver-derived mDC into allogeneic recipients induces IFNgamma-producing T cells (1).

Freshly-isolated liver DC differ phenotypically and functionally from DC isolated from other organs. DC reside in the liver under homeostatic conditions as "immature" APC (2, 3), expressing low levels of surface MHC class II, CD40, CD80 and CD86. Liver DC exhibit impaired Ag uptake compared to their splenic counterparts (3). They express lower levels of MHC class II and CD86 in response to maturation stimuli such as lipopolysaccharide (LPS) *in vitro*. Liver mDC, $CD8\alpha^+$ DC and pDC up-regulate CCR7 during maturation and migrate to lymphoid tissue (8). The migration route for liver pDC is controversial,- in rats, hepatic pDC are not found in the intestinal or hepatic lymph (9), suggesting that pDC may use alternative migration routes to reach T cells, or that liver pDC may activate T cells primarily within PALT. On the other hand, pDC are found in hepatic lymph nodes of healthy and diseased human livers (41), suggesting that liver pDC can migrate to regional lymphoid tissue in humans.

Freshly-isolated liver DC are poor stimulators of T cell proliferation compared to DC from the spleen (3, 42, 43). Murine liver $CD11c^+$ DC express lower basal levels of IL-12 (42) and, following either *in vitro* or *in vivo* LPS stimulation, secrete less IL-12 (42, 43). Liver mDC are more stimulatory in allogeneic T cell proliferation assays compared to liver pDC (13). Freshly-isolated mouse liver pDC secrete more TNFalpha and less IL-10 compared to spleen pDC (44). In response to viral infection, both liver and spleen pDC secrete IFNalpha, though this response is independent of both the receptor for viral CpG (TLR9) and the downstream signaling molecule, MyD88 in liver pDC (45). In humans, DC isolated after their *ex vivo* migration from liver tissue secrete elevated levels of IL-10, and are poor stimulators of T cell proliferation when compared to DC isolated from the skin in a similar manner (7).

Based on these observations, one might predict that the $CD4^+$ T cells in the liver are polarized towards at Th2 phenotype. Indeed, liver $CD4^+$ T cells analyzed *ex vivo* secrete elevated IL-4, IL-5 and IL-10, but also elevated IFNgamma compared to splenic $CD4^+$ T cells in response to TLR and CD28 ligation *in vitro*(46). *In vivo* studies of Ag-specific T cells reveal increased apoptosis of Th1 cells in the liver mediated by $CD11c^+$ DC, resulting in a Th2 bias within the liver (47). The down-regulated Th1 response induced by liver DC may play a role in allograft survival.

DC are potent inducers of Th17 cells, which have been reported to be both pro- and anti-inflammatory. Th17 cells secrete IL-22, that may act as an anti-inflammatory cytokine in the liver in response to LPS. IL-22 upregulates release of LPS-binding protein from hepatocytes (48) and is protective against acute liver injury in a murine hepatitis model (49, 50). The role of liver DC in the initiation of Th17 responses is yet to be discerned; this information may provide prove insightful in relation to liver tolerance.

The outcome of liver DC - T cell interactions may also be expansion/generation of either forkhead box P3 $(Foxp3)^+$ Treg or IL-10-secreting T regulatory type 1 (Tr1) cells. Tr1 cells are generated in response to high concentrations of IL-10, which are found in the liver. *In vitro*, mature human pDC induce Treg that are either transforming growth factor (TGF)beta-and IL-10-independent (51), or IL-10-dependent inhibitors of T effector cell proliferation (52). AlloAg-presenting pDC and Treg interactions in lymph nodes promote allograft survival in mice (14). Induction of liver allograft tolerance has been ascribed to Treg activity and liver rejection to Treg depletion (53). These findings contrast with a recent report suggesting that Treg are not required for liver allograft acceptance (54). Most recently, Foxp3 mRNA expression has been evaluated in biopsy samples from human liver transplant patients and found to be upregulated in those with acute rejection (55). However, in humans, expression of Foxp3 is not restricted to Treg, and is also found in activated $CD4^+$ T cells (56, 57), making these findings difficult to interpret in the context of tolerance.

3.4. Liver DC: relative resistance to LPS-induced stimulation

Cells in the liver are exposed continually to bacterial endotoxin (LPS) and other microbial products draining from the gut. LPS is a potent maturation stimulus

for DC and other APC. In spite of this, freshly-isolated liver DC are phenotypically immature, suggesting that they are resistant to maturation induced by LPS *in situ*. Freshly-isolated liver mDC and CD8alpha$^+$ DC express lower levels of mRNA for TLR4, the LPS receptor, compared to splenic mDC and CD8alpha$^+$ DC and are less able to induce allogeneic T cell proliferation, or polarize naïve T cells towards Th1 responses in the presence of LPS (43). This impaired responsiveness of liver DC to TLR4-mediated activation reflects a phenomenon known as 'endotoxin tolerance,' i.e. transient hyporesponsiveness to this molecule following previous exposure to LPS.

LPS in the liver may further downregulate immune responses initiated by other TLR ligands ('cross tolerance'). We have shown that IL-12 production by liver mDC is significantly lower than that of spleen mDC following ligation of the TLR9 receptor *in vitro* (42). In accord with this observation, CpG-activated liver mDC are less potent activators of allogeneic Th1 responses compared to CpG-activated spleen mDC. CpG-induced IL-12 production by liver mDC is further attenuated in the presence of LPS (42), suggesting that 'cross tolerance' may indeed modulate TLR responses in the liver.

Endotoxin tolerance and cross tolerance may be enhanced by the inherently high levels of IL-10, TGF-beta and IL-6 in the liver. IL-10 and TGF-beta are well-recognized modulators of T cell and APC function, and are produced by many cell types in the liver, including hepatocytes, KC and sinusoidal-lining endothelial cells in a type-I IFN dependent manner (57). Hepatic IL-10 is upregulated by LPS. This appears to be regulated by type-I IFN (58). pDC, that are comparatively abundant in the liver, are key producers of type-I IFN. However, the role of pDC in this model has yet to be evaluated. IL-6, found similarly in high concentrations, also appears to play a role in endotoxin tolerance in liver DC. A recent report showed that, while wild-type liver DC resist LPS-induced maturation, IL-6-deficient liver DC do not (59). This finding is supported by a recent study evaluating the influence of IL-6 on endotoxin tolerance and cross-tolerance. In this study, the concentration of TLR ligands was found to be an important factor determining the influence of IL-6 on DC maturation in response to TLR ligation (60).

LPS is elevated in the liver following IRI in rodents (61) and increased circulating LPS levels are associated with human liver transplantation (62). Therefore, the relative resistance of liver DC to LPS-induced maturation may affect allograft outcome. Mice deficient in the TLR signaling molecules, MyD88 (63, 64) or both MyD88 and TRIF (Toll/IL-1R domain-containing adaptor inducing IFNbeta) (64) accept skin grafts more readily than wild-type controls. Furthermore, exposure of the liver to soluble negative regulators of TLR signaling, such as ST2, decreases IRI (65), suggesting that further attenuation of TLR signaling may improve allograft outcome.

3.5. DC and liver transplant outcome

Donor liver-derived DC, a key constituent of the 'passenger leukocyte' population, have been implicated in the regulation of alloimmune reactivity and liver transplant outcome (66-68). DC of donor origin can be propagated from the bone marrow of mice that accept liver allografts without immunosuppressive therapy, but not from recipients of heart allografts from the same donor strain that acutely reject their grafts (66). Liver-derived DC progenitors have been shown to induce Th2 cytokine-producing cells in secondary lymphoid tissue of allogeneic recipients (40), and to prolong pancreatic islet allograft survival (38).

The balance between potential immunostimulatory and tolerogenic DC function in the liver may be an important predictor of transplant outcome. Mobilization of DC from the bone marrow into donor mouse livers by fms-like tyrosine kinase 3 ligand (Flt3L) administration before transplant augments rejection (69, 70), that is reversed by host treatment with anti-IL-12 mAb (71). Treatment of mice with Flt3L boosts the production of large numbers of liver DC progenitors (72), and increases the numbers of mDC, CD8alpha$^+$ DC and pDC in the liver (1, 44). It has been reported (42, 44, 69) that mDC and pDC from these livers exhibit increased activation markers, such as MHC II, CD80 and CD86, and enhanced allogeneic T cell stimulatory capacity, that may promote rejection, compared with hepatic DC from untreated mice. In spite of this, the T cell allostimulatory capacity of freshly-isolated or LPS-activated bulk CD11c$^+$ DC isolated from the livers of Flt3L-treated mice remains inferior when compared to that of splenic mDC from the same animals (42, 43).

Rejection of Flt3L-treated mouse donor livers (as opposed to 'spontaneous' acceptance of normal allogeneic livers) may result from enhanced Th1-polarizing ability of the liver DC following their *in vivo* mobilization (1). Allograft rejection of livers from Flt3L-treated donor mice may contravene endotoxin tolerance. While hepatic DC from Flt3L-treated animals exhibit characteristics of endotoxin tolerance, such as diminished IL-12 secretion following stimulation with LPS or CpG (42)(43), Flt3L treatment following the development of systemic endotoxin tolerance reverses the tolerant state (73). These studies suggest that conditioning of DC in the liver, rather than their mobilization to this site, is necessary for tolerance. As such, characterization of intracellular signaling pathways in hepatic DC may reveal molecular events necessary for the potentiation of DC-mediated allograft tolerance.

In humans, examination of the relative incidences of circulating pDC versus mDC precursors in the peripheral blood of liver allograft recipients has revealed a higher pDC:mDC ratio in tolerant patients and in patients on progressive, immunosuppressive drug weaning, compared with those on maintenance immunosuppression (MI) (74). These differences could not be ascribed to the levels of immunosuppressive drug therapy (75). Moreover, elevated expression of PD-L1 relative to CD86 on the surface of pDC in the tolerant patients correlated with a higher incidence of circulating Foxp3$^+$ CD25$^+$ CD4$^+$ Treg compared with patients on MI (76).

4. MOLECULAR REGULATION OF LIVER DC SIGNALING/MATURATION

The reduced sensitivity of liver DC compared to those from other peripheral organs to stimulation with most PAMP molecules suggests that signaling through pattern recognition receptors (PRR) may also be uniquely regulated in liver DC. Altered PRR signaling also likely contributes to the phenotype of liver DC. However, compared to *in vitro*-generated DC, or DC from other organs, PRR signaling in liver DC has not been studied in detail.

While several families of PRR are known, only the well-known TLR family has been studied in liver DC. Both liver and spleen $CD11c^+$ DC express TLR 1-9 mRNA (77). However, expression of individual TLRs differs between liver and spleen DC. For instance, expression of TLR4 mRNA is lower in liver compared to spleen DC (43, 77), while TLR9 mRNA or protein expression is similar between DC from these organs (42, 77). Moreover, liver DC TLR expression can differ among DC subsets. Thus, while both liver and spleen $CD11c^+$ DC express quantifiable amounts of TLR1-9 mRNA, liver $B220^+CD205^+$ DC express mRNA for only TLR1, 2, 6, 7, and 9, with minimal or no transcripts for other TLR molecules (77). The liver $B220^+CD205^+$ DC exhibit greater TLR9 mRNA expression compared with both liver and spleen $CD11c^+$ DC (77).

Several intracellular mechanisms inhibit TLR signaling via the MyD88-IRAK (IL-1R-associated kinase)-TRAF6 (TNFR-associated factor 6) pathway. These include MyD88s, a splice variant of MyD88, ST2, IRAK-M, and SIGIRR (single immunoglobulin domain IL-1R-related), many of which have been associated with endotoxin tolerance (78). However, only IRAK-M has been examined in liver DC. Lunz *et al* (59) showed that IRAK-M mRNA expression was increased significantly in liver DC and liver tissue from IL-$6^{+/+}$ compared to IL-$6^{-/-}$ mice. Expression of IRAK-M is known to be elevated in macrophages exposed continually to endotoxin, and similar exposure to endotoxin occurs in the liver as the result of its perfusion with gut-derived portal blood (79). More detailed examination of PRR signaling may provide roles for these and other negative PRR regulators in determining the differences in PAMP sensitivity between liver DC and those from other organs.

As with the dearth of information concerning intracellular PRR signaling in liver DC, few of the other intracellular signaling cascades that have been well-characterized in non-hepatic DC maturation and function, have been studied in liver DC. Lunz *et al* (59) have described recently how elevated STAT3 (signal transducer and activator of transcription 3) signaling in the liver compared to other organs contributes to liver DC hyporesponsiveness (59). In this study, gut-derived commensal bacterial products were shown to drive hepatic IL-6 production and activation of STAT3, thereby inhibiting liver DC maturation and function. Moreover, IL-$6^{-/-}$ liver DC displayed a greater maturation response to bacterial PAMP molecules than wild-type IL-$6^{+/+}$ liver DC. Suppression of liver DC activation by STAT3 activity is a similar mechanism to that in cancer, where constitutive STAT3 activation inhibits DC maturation and immune recognition of tumor cells (80, 81).

IL-6/STAT3 signaling may prevent DC maturation through an antagonistic relationship with nuclear factor (NF)kappaB, a common transcription factor activated during DC maturation (82). DNA binding sites for activated STAT3 and NFkappaB are known to overlap and DNA binding of STAT3 can prevent the transcription of genes, such as α2 macroglobulin (83). It is unknown whether NFκB and STAT3 DNA binding consensus sequences overlap in genes necessary for DC maturation. However, diminished phosphorylation of STAT3 in LPS-treated IL-$10^{-/-}$ DC allows increased recruitment of the NFkappaB c-Rel subunit to the IL-12 promoter, a cytokine expressed typically by mature DC (84).

5. DC-NK/NKT CELL INTERACTIONS: AN EMERGING AREA OF LIVER IMMUNOBIOLOGY

The normal liver lymphocyte population comprises ~30% $CD3^-CD56^+$ NK and 40% TCR V alpha 24^+CD56^+ NKT cells, a significantly greater percentage compared to that found in the circulation and secondary lymphoid organs (85, 86). Despite the comparatively high incidence of these cells, DC interactions with NK and NKT cells in the liver are poorly-described, especially in the context of tolerance and transplantation.

The regulatory ability of NKT cells has implicated these cells in the promotion of transplant tolerance (87, 88). NKT cells comprise a T cell subset that responds to glycolipid Ag presented on CD1d molecules. Their activation results in production of Th1 or Th2 cytokines and subsequent activation of B, T, and NK cells, and DC (89, 90). NKT cell activation can also trigger Fas- or perforin-dependent cytotoxic effects (91, 92). Since CD1d is expressed on numerous liver cell types, including DC and hepatocytes, this provides ample opportunity for NKT cell activation (93, 94).

The majority of NKT cell studies are performed using DC loaded with alpha galactosyl ceramide (alphaGC), a non-physiologic ligand for NKT cells (95). Stimulation of NKT cells with alphaGC induces the production of both Th1 and Th2 cytokines, and may not accurately depict their function during normal physiologic immune responses (96, 97). However, one group has shown (98) that repeated stimulation of NKT cells with alphaGC in mice induces IL-10-producing, regulatory DC with an immature phenotype, reduced NFkappaB DNA-binding activity, and ability to inhibit immune responses *in vivo*. The recent identification of myelin-derived 3'-sulfated GC (sulfatide) as a natural ligand for NKT cells may provide a more physiologic stimulus for studying NKT cell regulation of immune responses (99, 100).

Despite the relative lack of data concerning the response of NKT cells to endogenous ligands, it has been

reported that culture of NKT cells with $CD1d^+$ APC in the absence of exogenous ligand skews the NKT cell response toward an IL-5/IL-13- producing Th2 phenotype (101). Therefore, the abundance of NKT cells and the expression of CD1d on liver APC could provide continuous suboptimal stimulation with endogenous ligands, leading to Th2 regulation of inflammation that may contribute to the tolerogenic properties of the liver.

NKT cell activation by alphaGC-loaded DCs results in IFNgamma and IL-12 production by NKT cells and DC, respectively. These cytokines can activate NK cells synergistically (102). Additionally, indoleamine 2,3-deoxygenase (IDO), which is produced by activated pDC, has been shown to promote NK cell function (103, 104). Typically described for their anti-tumor and anti-viral cytolytic function, NK cells have also been implicated in promoting allograft tolerance through the direct lysis of donor-derived APCs from skin transplants (105). In addition, interaction of NK cells with hepatocytes results in priming of DC, leading to the induction of a regulatory T cell population that suppresses $CD4^+CD25^-$ T cell responses through a programed death (PD)-1 dependent mechanism (106). These findings provide evidence for DC-NK cell interactions that may contribute to the induction of tolerance in the liver.

The interaction of pDC with NKT cells represents another mechanism that may help to explain the tolerogenic properties of the liver. In mice, presentation of sulfatide to Type II NKT cells by pDCs in the liver results in reciprocal activation of pDCs and recruitment to the liver of invariant NKT (iNKT) cells which are anergic and prevent inflammatory liver disease through an IL-12-dependent mechanism (100) This provides direct evidence that pDC interaction with NKT cells in the liver can induce a tolerogenic phenotype upon activation by self glycolipids.

Although human pDC do not express CD1d, they express other molecules and produce cytokines that have been reported to interact with and activate NKT cells. These include glucocorticoid-induced TNF receptor ligand (GITRL), ICOSL, OX40 ligand TNF and type I IFNs (107-110). The function of these molecules in regard to NKT cell activation appears to be a costimulatory role; however, their role in the induction of tolerance remains unclear. Interestingly, it has been reported that CpG activation of human peripheral blood pDC is required for NKT cells to respond to alphaGC presentation on CD1d by mDC (111). Together, these data suggest a potential role for pDC in regulating the activation of NKT cells by other DC subsets.

Viral infections represent a substantial threat to allograft survival (112-114). This may be due to the negative effects of post-transplant immunosuppressive drug therapy on DC, as well as on the ability of the innate immune system to respond to and clear viral infections (115-118). Reduced DC availability and function may lead to impaired interaction with NK and NKT cells, consequently limiting their ability to control viral infections (119). Clearly, the roles for DC-NK/NKT cell interactions during viral infection following transplantation warrant further investigation. Despite evidence linking DC-NK/NKT cell interactions, tolerance and transplantation, the field remains largely unexplored and open to new interpretations.

6. LIVER REGENERATION AND DC FUNCTION

It is well-known that the liver can regenerate following injury; this process is central to the success of surgical resection and live-donor liver transplantation. Many factors are believed to be involved in regulation of liver regeneration (120) and innate and adaptive immune responses are thought to play a key role. Several observations suggest that, during liver regeneration, the hepatic microenvironment promotes a transient, tissue-specific, immunosuppressed state. Vujanovic *et al* (121) demonstrated strong inhibition of the cytotoxic activity of liver-resident NK cells against proliferating hepatocytes soon after partial hepatectomy (PH), with restoration of their function at the end of the regenerative process (121). In a murine model of liver regeneration after PH, a temporary increase in the number of immature liver DC has been reported (122). In this model, liver DC acquired tolerogenic properties: up-regulated IL-10, down-regulated IFNgamma gene transcription and induction of Th2 cytokine production (high levels of IL-10 and low levels of IFNgamma production) by naïve allogeneic T cells. Interestingly, these functional changes were concomitant with an increase in expression of estrogen receptors by liver DC and high serum levels of 17beta-estradiol (a typical finding during liver regeneration after PH) (123, 124). Recently, there have been several reports that estrogen administration leads to clinical improvement in experimental autoimmune encephalomyelitis (EAE), due to changes in DC function and the promotion of a Th2 immune response (125-127). These observations suggest that, during liver regeneration, estrogen-exposed DC may play a role in local immunosuppression, by altering the balance toward a Th2-like microenvironment, as shown in EAE. Suppression of NK cell function and reduction in IFNgamma production during hepatocyte proliferation have also been reported (128) in a rat model of post-transplant liver regeneration. The authors demonstrated that infusion of NFkappaB decoy oligodeoxynucleotide-modified DC (unable to undergo maturation, and with tolerogenic potential) increased regeneration of the liver graft. In addition, the enhanced regenerative ability of the resected liver following *in vivo* expansion of DC by Flt3L administration (122), further suggests a role for these cells in supporting hepatocyte proliferation, possibly skewing tissue-specific Ag immune responses towards a tolerogenic state. Moreover, this positive relation between liver regeneration and immunosuppression has been confirmed in a murine model in which administration of immunosuppressants, such as tacrolimus or cyclosporine, increases hepatocyte proliferation after PH (124, 129).

IRI during organ retrieval and transplantation is associated with the release of proinflammatory cytokines and chemokines, and the recruitment of DC and other leukocytes to the liver (130). In this context, liver regeneration plays a pivotal role in the healing process and

graft survival. Loi *et al* (131) have shown that, soon after IRI in a mouse model, liver DC increase in number, especially around and within necrotic areas. Even although they exhibit a mature phenotype, these DC produce immunosuppressive cytokines (IL-10 and TGF-beta), a finding in keeping with polarization towards Th2 cytokine production by liver DC during liver regeneration after PH (122). Others, however, have shown that DC can participate in IRI damage by promoting pro-inflammatory events (132).

7. INFLUENCE OF VIRAL HEPATITIS AND LIVER CANCER ON DC FUNCTION

7.1 Viral hepatis and impairment of DC function

HCV- or HBV-related liver failure is a common indication for liver transplantation worldwide, and graft re-infection post-surgery is an important clinical problem, especially for HCV-infected patients, for whom no effective prophylaxis is available. HCV- or HBV-related hepatitis represents another condition in which the outcome depends on the balance between immunoreactivity and immunosuppression/tolerance. After viral infection, activated DC induce the differentiation of naïve T cells into virus-specific $CD4^+$ (IFN-gamma; anti-viral Ab) and $CD8^+$ T cells for adaptive immunity (133). These events should control and eliminate virus infection. Unfortunately, in most HCV and a significant number of HBV infections, the immune response fails to eliminate the virus. In the past few years, several authors have demonstrated functional impairment of DC in HCV-infected patients. Nattermann *et al* (28) have shown a decreased number of $BDCA1^+$ and $BDCA2^+$ DC in peripheral blood of HCV-infected patients, together with a concomitant increase in their number in liver tissue. The authors suggest that the interaction of the HCV E2 Ag with CD81, a member of the tetraspanin family (considered the cellular receptor for entry of HCV expressed on DC), could result in impaired migration of DC towards the chemokine CCL21, which modulates DC trafficking from sites of infection to lymphoid tissue, with consequent impairment of T cell priming. In addition, decreased CD86 expression and IL-12 secretion by DC from chronic HCV-infected patients has been detected, along with impaired allogeneic T cell stimulatory activity of mDC (134) and reduced IFNgamma (135) and increased IL-10 (136) secretion by T cells. Tsubouchi E *et al* (137) have reported that the functional impairment of DC in HCV-infected patients may be related to the presence of HCV-RNA in both mDC and pDC.

With regard to HBV infection, several studies in mice and humans have shown that the infection induces decreased T cell stimulatory activity of DC (138, 139) and the generation, by pDC, of a higher proportion of $CD4^+$ $CD25^+$ Treg (140). Recently, however, Tavakoli *et al* (141) have shown no quantitative, phenotypic or functional impairment of mDC or pDC in chronic HBV carriers.

Immunosuppressants such as corticosteroids, calcineurin inhibitors, and rapamycin, usually used to inhibit graft rejection by preventing DC maturation and T cell activation/proliferation, increase the risk for HCV-related disease (142). In addition, Schvoerer *et al* (118) have shown a significant decrease in circulating pDC and mDC 7 days after liver transplantation, probably due to their migration into the liver during the first days following transplantation; such DC kinetics could be related to HCV graft reinfection.

7.2 Liver cancer, functional modification of DC and immunotolerance

Liver transplant is a treatment option for hepatocellular carcinoma (HCC). Functional modification of DC and Treg is believed to play a critical role in inducing immune tolerance to tumor Ag in hepatocellular carcinoma (HCC) patients. An increased prevalence of Treg in both peripheral blood and tumor tissue, able to actively suppress proliferation of autologous circulating $CD4^+CD25^-$ and $CD8^+$ T cells (143, 144), has been reported in HCC. Immature-semi-mature DC have been shown to induce the development of Treg (145), and there is growing evidence of an immature phenotype of DC, both in tumor tissue and peripheral blood of HCC patients. Ninomiya *et al* (146) have shown that monocyte-derived DC in HCC exhibit reduced levels of HLA-DR and impaired IL-12 production and T cell allostimulatory capacity. These findings are associated also with an increased level of IL-10 and reductions in circulating mDC and pDC (147). Recently, Li *et al* (148) have demonstrated that tumor culture supernatant from hepatoma-derived cell lines inhibits the differentiation of monocytes into DC, with retention of CD14 and reduced expression of CD1a. Moreover, in this model, tumor culture supernatant-exposed DC increased IL-10 secretion and failed to mature after LPS stimulation. Taken together, these findings suggest how the tumor microenvironment may promote mechanisms able to induce tumor immunotolerance.

8. IMPACT OF ANTI-INFLAMMATORY AND IMMUNOSUPPRESSIVE AGENTS ON DC FUNCTION

It is understood that clinical immunosuppressants target T and B lymphocytes through blockade of Ag receptors or other critical signaling pathways needed for full cell activation. However, there have been few assessments of the influence of immunosuppressants on liver DC, or for that matter, non-lymphoid tissue DC, in general. Nevertheless, based chiefly on studies of human and murine DC generated *ex vivo*, murine splenic DC, or circulating human DC precursors, it is apparent that immunosuppressive agents, by targeting common signaling components shared between leukocytes, exert profound immunomodulatory effects on the development and function of DC (149, 150). Several commonly-used clinical immunosuppressive/anti-inflammatory drugs, including corticosteroids, cyclosporine, tacrolimus, deoxyspergualin, mycophenolate mofetil, acetylsalicylic acid, and rapamycin, can block the immunostimulatory function of DC (149, 151). Generation of DC in the presence of these agents characteristically results in cells of reduced maturity, expressing low levels of co-stimulatory molecules, especially CD86, that elicit poor production of Th1 cytokines, including IL-12, TNF-alpha, and IFN-gamma.

The majority of pharmacologic agents examined impede activation of NFkappaB, a central regulator of DC maturation and a systemic controller of cellular inflammatory responses (152). Pharmacologically-modified DC display resistance to maturation upon encountering inflammatory stimuli, and have propensity to induce anergy/apoptosis in responsive naïve effector T cells, even under inflammatory conditions. These DC, however, are well-suited to enrich for generate Treg and, when assessed as 'negative' vaccines for transplant tolerance, can induce long-term experimental allograft survival, which is often associated with Treg activity (153, 154).

In addition to the above immunosuppressive and anti-inflammatory agents, IL-10, TGFbeta, and LPS, all highly-enriched in the liver microenvironment, have been employed successfully to instill tolerogenic/immunosuppressive characteristics on DC during their the *ex vivo* generation (155-159). Specifically, "alternatively-activated" (AA) DC generated *ex vivo* in GM-CSF, IL-10, TGFbeta, and then exposed to LPS, display low levels of CD40, CD80, CD86, and produce little IL-12. Similarly to DC treated with corticosteroids, they have greatly increased expression of IL-10. Importantly, AADC generated under conditions related to those found in the liver, are poor stimulators of T cells, and induce Ag-specific hyporesponsive effector T cell responses, while directing the expansion/generation of functional Treg (155, 158, 159). In addition, infusion of AADC protects mice from lethal endotoxemia and peritonitis (157) and graft-versus-host-disease (155, 159) and, when combined with CTLA4-Ig, promotes indefinite murine allograft survival (158).

1alpha,25$(O)_2D_3$,- the active metabolite of vitamin D_3, is generated specifically in the liver by cytochrome p450 enzymes (25-hydroxylases; 12) and potently inhibits DC differentiation and maturation (160, 161) . DC express the vitamin D receptor and 1alpha,25$(O)_2D_3$ treatment of DC impairs the expression of CD40, CD80, CD86 and IL-12. It also inhibits the phosphorylation and nuclear translocation of NFkappaB, while significantly increasing IL-10 production (151). As with other immunosuppressant-modified DC when infused into murine transplant models, 1alpha,25$(O)_2D_3$-treated DC stimulate Treg and promote transplant tolerance (162). Intriguingly, it has been demonstrated recently that human mDC themselves can metabolize vitamin D to 1alpha,25$(O)_2D_3$ and, as a result, modify T cell function (163). Furthermore, the modulation of human DC by 1alpha,25$(O)_2D_3$ appears to be highly selective, in that only mDC, and not pDC, display inhibited function (164). Specifically, while mDC were potently inhibited by treatment with 1alpha,25$(O)_2D_3$, similarly-treated pDC did not exhibit any discernible inhibitory effects, having unimpaired NFkappaB activation and maintaining their potent production of IFN-alpha. Thus, 1alpha,25$(O)_2D_3$ is similar to the glucocorticoid dexamethasone, which, although it reduces both mDC and pDC numbers in the spleen and liver of mice, does not inhibit pDC production of IFNalpha (117). Although preliminary, these findings suggest that pharmacologic immunosuppressants, and possibly the liver environment, do not modulate DC subsets equally. Further study of the specific effects of these immunosuppressants and liver-produced factors on pDC in particular (since these cells are more abundant in the liver compared with spleen, but relatively understudied compared to mDC) may yield critical insights into the basic immunobiology of peripheral tolerance. Also, further elucidation of the shared and unique factors/mechanisms by which pharmacologic immunosuppressants and the liver environment instill tolerogenic capacity upon various DC may yield potential therapeutic targets/mechanisms. By acquiring such knowledge, strategies may evolve to allow immunosuppressant treatment to act synergistically with the inherent tolerogenic properties of liver DC.

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Abbreviations: APC, antigen-presenting cell; CpG, cytidylyl phosphate guanosine; DC, dendritic cell(s); Flt3L, fms-like tyrosine 3 kinase ligand; HCV, hepatitis C virus; IFN, interferon; IRI, ischemia-reperfusion injury; KC, Kupffer cell; CPS, lipopolysaccharide; mDC, myeloid DC; NF (kappaB), nuclear factor; NK, natural killer; PALT, portal-associated lymphoid tissue; pDC, plasmacytoid DC; PH, partial hepatectomy; PRR, pattern recognition receptors; TGF(beta), transforming growth factor; Th, T helper; TLR, Toll-like receptor; Treg, regulatory T cell.

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