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Graft-infiltrating PD-L1^{hi} cross-dressed dendritic cells regulate anti-donor T cell responses in mouse liver transplant tolerance

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Abstract

While a key role of cross-dressing has been established in immunity to viral infection and more recently in the instigation of transplant rejection, its role in tolerance is unclear. Here, we investigated the role of intra-graft dendritic cells (DC) and cross-dressing in mouse major histocompatibility complex (MHC)-mismatched liver transplant tolerance that occurs without therapeutic immunosuppression. While donor interstitial DC diminished rapidly following transplantation, they were replaced in the liver by host DC that peaked on postoperative day (POD) 7 and persisted indefinitely. About 60% of these recipient DC displayed donor MHC class I, indicating cross-dressing. By contrast, only a very minor fraction (0–2%) of cross-dressed DC (CD-DC) was evident in the spleen. CD-DC sorted from liver grafts expressed much higher levels of T cell inhibitory programmed death ligand 1 (PD-L1) and high levels of IL-10 compared with non CD-DC (nCD-DC) isolated from the graft. Concomitantly, high incidences of programmed death protein 1 (PD-1)^{hi} T cell immunoglobulin and mucin domain containing-3 (TIM-3)⁺ exhausted graft-infiltrating CD8⁺ T cells were observed. Importantly, unlike nCD-DC, the CD-DC failed to stimulate proliferation of allogeneic T cells but markedly suppressed anti-donor host T cell proliferation. CD-DC were much less evident in allografts from DNAX-activating protein of 12kDa (DAP12)^{-/-} donors that were rejected acutely.

Conclusion—These findings suggest that graft-infiltrating PD-L1^{hi} CD-DC may play a key role in the regulation of alloimmunity and in the induction of liver transplant tolerance.

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Keywords

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Introduction

The liver is considered the most tolerogenic of transplanted organs. Thus, fully MHC-mismatched liver grafts are accepted between mouse strains without immunosuppressive therapy and induce robust, donor-specific tolerance.¹ In humans, unlike with other organs, approximately 20% of stable liver transplant recipients can be weaned safely off all immunosuppression.² However, the mechanisms underlying liver transplant tolerance are not well understood. Better understanding of these mechanisms could have important implications for the promotion of tolerance in the clinic and reduced dependence of transplant recipients on immunosuppressive drugs. Importantly, the liver has a unique constituency of parenchymal and non-parenchymal cells (NPC) that have been implicated in immune regulation and tolerance induction.^{3, 4} These include resident professional and non-professional antigen (Ag)-presenting cells (APC), i.e. DC and hepatic macrophages (Kupffer cells),^{5, 6} liver sinusoidal endothelial cells (LSEC),⁷ hepatic stellate cells⁸ and hepatocytes.⁹ Of these, DC are uniquely well-equipped APC that promote self-tolerance in the steady-state¹⁰ and regulate immunity.¹¹ Liver-resident DC comprise several subsets^{4, 5} characterized by their immaturity, resistance to maturation¹² and tolerogenic properties.^{13–15} The predominant, conventional liver myeloid DC population has been shown to attenuate hepatic inflammation and fibrosis,^{16, 17} and to regulate liver warm and transplant-induced ischemia-reperfusion injury.^{18, 19} Moreover, liver DC can subvert T cell responses^{5, 13, 15, 20} and prolong allograft survival.^{21, 22}

Molecular mechanisms whereby liver APCs regulate/inhibit T cell responses include their expression of programmed death ligand-1 (PD-L1= B7 homologue 1 [B7-H1])^{7, 15, 23}, IL-10,²⁴ FasL,²⁵ the Notch ligand Jagged 1,⁹ CD39¹⁹ and the immunoreceptor tyrosine-based activation motif (ITAM)-bearing transmembrane adaptor protein DNAX-activating protein of 12kDa (DAP12).^{26, 27} Of these molecules, PD-L1, IL-10 and DAP12 have been implicated in regulation of mouse ‘spontaneous’ liver transplant tolerance.²²

An important role of cross-dressing of host APC has been established in immunity to viral infection,²⁸ and more recently in the acute rejection of murine kidney, heart and skin grafts^{29, 30}. Thus, Liu et al²⁹ have shown that, in mouse heart transplantation, host DC that acquire donor intact MHC molecules (cross-dressed DC; CD-DC) play an important role in allograft rejection. MHC molecules transferred from donor DC via extracellular vesicles were internalized or remained attached to recipient DC and promoted their activation. This cross-dressing by host DC may occur throughout the life span of the graft.³¹ Given these observations, and since mechanisms underlying spontaneous liver allograft acceptance remain unclear, we examined the properties of graft-infiltrating CD-DC in the mouse orthotopic liver transplant model. Our data establish a new role for cross-dressing in transplant tolerance and indicate that this mechanism may underlie the acceptance of hepatic allografts in the absence of immunosuppressive therapy.

Materials and Methods

STUDY DESIGN

For all (liver transplant) experiments, age-matched animals were distributed randomly into experimental groups. Liver transplants were performed from male B6 donors to male C3H recipients since for anatomic/surgical reasons, male B6 (or B10) into male C3H is the model of choice for orthotopic allogeneic liver transplantation in the mouse.^{27, 32–34} After surgery, each mouse was housed alone for the first week. All surgeries were performed during the light cycle, without fasting. The number of animals (3–6 mice per time point post-transplant) used in each analytical experiment was based on previous studies in which the orthotopic allogeneic liver transplant model has been used. All experiments were conducted under an Institutional Animal Care and Use Committee-approved protocol and in accordance with criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

MICE

Male C57BL/6 (B6; H-2^b), B6 SJL-PtprcaPepcb/BoyJ (B6 SJL CD45.1), B6.Cg-Tg(Itgax-Venus)1Mnz/J (CD11c-yellow fluorescent protein [YFP]) and C3H/HeJ (C3H; H-2^d) mice (8 to 12 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Male DAP12^{−/−} mice,³⁵ generated initially in the 129/SvJ and B6 hybrid background, were backcrossed onto B6 and breeding pairs kindly provided by Dr. Marco Colonna, Washington University School of Medicine, St. Louis, MO. Animals were maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh School of Medicine. They were fed a diet of Purina rodent chow (Ralston Purina, St. Louis, MO) and received tap water ad libitum. Mice (4–5 per cage) were housed in Optimice® (Animal Care Systems, Inc., Centennial, CO).

LIVER TRANSPLANTATION

Orthotopic mouse liver transplantation was performed as described in detail,³⁴ without immunosuppressive therapy.

FLOW CYTOMETRY AND FLOW IMAGING

Cells were incubated with Zombie Aqua™ dye (Zombie; Biolegend, San Diego, CA) according to the manufacturer's instructions and treated with FcγR-blocking rat α-mouse CD16/32 Ab (93; Biolegend). For surface staining, cells were incubated for 30 min at 4°C with different combinations of fluorochrome-conjugated Ab specific for H-2K^b (AF6-88.5), H-2D^k (AF6-120.1), CD3 (PB 17A2), CD4 (H129.19), CD8α (53-6.7), CD11c (HL3), CD11b (M1/70), CD45.1 (A20), CD45.2 (104), CD44 (IM7), CD49b (DX5), CD62L (MEL-14), CD80 (16-10A1), CD86 (GL1), PD-L1 (CD274; 10F.9G2), B220/CD45R (RA3-6B2), F4/80 (BM8), NK1.1 (PK136), TIM-3 (8B.2C12) or PD-1 (J43) (eBioscience, BD, Biolegend). After staining, the cells were fixed in 4% v/v paraformaldehyde. For intracellular staining, the cells fixed and permeabilized using Fix and Perm reagent (eBioscience) and the following fluorochrome-conjugated Abs specific for: CTLA-4

(UC10-4B9), IFN γ (XMG1.2), TNF α (MP6-XT22), IL-2 (JES6-5H4), IL-6 (MB5-20F3), IL-10 (JES5-16E3) or IL-12p40 (C17.8) (eBioscience, BD) used. Appropriate fluorochrome-conjugated isotype-matched IgG was used as a negative control. Before cytokine staining the cells were cultured for 4 h at 10^6 cells/ml in 15 ml tubes with phorbol myristate acetate (PMA; 20 ng/ml, Sigma) and ionomycin (100 mM, Sigma). GolgiStopTM (0.66 μ l/ml; BD) was added 30 min after PMA/ionomycin addition. Annexin-V Apoptosis Detection Kit was purchased from eBioscience and used according to the manufacturer's instructions.

All flow data were acquired on a LSR Fortessa flow cytometer (BD) and analyzed using FlowJo v10 software (Tree Star, San Carlos, CA). Imaging cytometry cell data were acquired on an ImageStream IS100 (Amnis Corporation, San Diego, CA) and analyzed using IDEAS software (Amnis).

FLOW SORTING

Graft NPC were isolated on post-operative day (POD) 7. CD11c⁺ selection was performed by MACS (Miltenyi), followed by H-2K^b positive and negative flow sorting (CD-DC and nCD-DC) using a BD FACS Aria II (San Diego, CA).

NANOSTRING ANALYSIS

A 561-gene panel (mouse immunology panel; www.nanostring.com) including 14 housekeeping genes and six positive controls (Nanostring Technologies, Seattle, WA) was used. RNA was extracted from flow-sorted graft CD-DC or nCD-DC using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA concentrations were determined by Nanodrop (Thermo Scientific, Wilmington, DE). Reporter and capture probes were hybridized during 20 h incubation at 65 °C and the resulting RNA complexes subsequently immobilized and counted on an nCounter analyzer (Nanostring Technologies) according to the manufacturer's instructions. Raw data were normalized based on the geometric mean of negative controls, internal housekeeping genes and positive controls in nSolver 3.0. Ingenuity pathway analysis (IPA) software (Qiagen) was used for pathway analysis.

CONFOCAL MICROSCOPY

After FACS, CD-DC were re-stained with CD11c, H-2K^b and H-2D^k Ab, then fixed with 4% paraformaldehyde; confocal microscopy was performed using a Nikon A1 confocal microscope.

TWO-PHOTON INTRAVITAL MICROSCOPY

Two-photon intravital microscopy was performed on native livers of CD11c-YFP mice and on CD11c-YFP liver allografts (on POD 7 and 30). Mice were anaesthetized with isoflurane and the liver exposed after a midline abdominal incision. The left lateral lobe was pulled gently and immobilized with a custom cup for intravital imaging. Oxygen and core body temperature were maintained at 37°C with a homeothermic controller (TC-1000, CWE, Ardmore, PA). Blood vessels were visualized by injecting Evans Blue (100 μ g in PBS i.v.). Image acquisition was performed using an A1R MP Nikon system and a Ti:Sapphire Chameleon IR (Coherent) laser tuned and mode-locked to 900 nm.³⁶

STATISTICAL ANALYSIS

GraphPad Prism (version 5.00; Graphpad Software Inc., San Diego, CA) was used for statistical analyses. Results are expressed as means $\pm$ SEM. Multiple comparisons on a single data set were performed by 1-way ANOVA, followed by Tukey-Kramer multiple comparisons test. In all experiments, $P < 0.05$ was considered significant.

ADDITIONAL METHODS

Details of additional methods are provided in the Supporting Information.

Results

HOST DC CROSS-DRESSED WITH DONOR MHC MOLECULES (CD-DC) ARE PREVALENT IN LIVER ALLOGRAFTS

To determine the source of DC in liver allografts, we first examined the presence of donor- and recipient-derived DC in B6 (H-2^b) grafts of C3H (H-2^k) recipients at various time points post-transplant (Fig. 1A). No immunosuppressive therapy was administered; in this model >80% of liver grafts survive long-term (>100 days).^{27, 33} DC were defined as CD11c⁺, CD3⁻, NK1.1/CD49b⁻, and B220⁻ cells. By flow cytometric analysis, donor DC (expressing donor, but not recipient MHC class I) gradually disappeared and were not detected in the graft or spleen by post-operative day (POD) 7. Recipient DC rapidly infiltrated the grafts. Interestingly, the percentage of recipient DC cross-dressed with donor MHC class I (Fig. 1B), increased progressively over time. To confirm that donor DC disappeared from the graft shortly after transplantation, and to investigate whether donor hematopoietic cells or other liver-resident cells (hepatic stellate cells, LSEC, cholangiocytes or hepatocytes) were the source of cross-dressing, we examined grafts transplanted from B6 CD45.1 donors to C3H (CD45.2) recipients. MHC class I is expressed by all nucleated cells, while CD45 is only expressed by hematopoietic cells. As shown in Fig. 1C, donor CD45.1⁺ DC disappeared by POD 7. In this model, recipient DC did not acquire CD45.1 molecules, which suggests that donor non-hematopoietic cells (CD45.1⁻) were the main source of cross-dressing.

To increase the sensitivity of donor cell detection, we also examined B6 yellow fluorescent protein (YFP)-CD11c liver allografts transplanted into C3H recipients by 2 photon intravital microscopy. While some donor CD11c⁺ YFP⁺ cells remained in the graft on POD 7, these cells disappeared completely by POD 30 (Fig. 1D). Thus, donor DC disappear from mouse liver allografts rapidly and are replaced by host-derived graft-infiltrating DC, the majority of which are cross-dressed with donor MHC class I.

Other host-derived (CD45.2⁺) leukocytes were also cross-dressed with donor intact MHC class I molecules. As shown in Fig. 1E and F, on POD 7, 25.8 % of CD45⁺ live cells were cross-dressed, and within this population, 33.3 % were CD11c⁺, 15.9 % CD11c⁻ CD11b⁺ F4/80⁺, 23.8 % CD11c⁻ CD11b⁺ F4/80⁻, and 15.7 % were T cells. Given the professional Ag-presenting function of CD11c⁺ DC, their perceived role in liver tolerance, and the prevalence of CD-DC in the graft, we focused further studies on this key immune cell population.

HEPATOCYTES ARE THE PRINCIPAL SOURCE OF DONOR MHC CLASS I EXPRESSED BY CD-DC

To determine which hepatic parenchymal cell(s) were the source of donor MHC class I expressed by host DC, we co-cultured C3H (host strain) DC for 20h with either purified normal B6 liver hepatocytes, LSEC or hepatic stellate cells for 20 hr, in the absence or presence of 0.4µm transwells. The DC were then examined by flow cytometry for the expression of MHC class I of both the donor (H-2K^b) and 'acceptor' (H-2D^k) strains. As shown in Fig. 2, DC co-cultured with hepatocytes showed clear evidence of cross-dressing (mean 15% CD-DC), whereas much lower levels (~ 5%) of DC were cross-dressed with donor MHC class I from LSEC or stellate cells. The presence of transwells markedly reduced the incidence of DC that expressed hepatocyte-derived donor MHC class I, indicating that physical contact was required for cross-dressing.

CD-DC IN LIVER ALLOGRAFTS EXPRESS HIGHER PD-L1 AND IL-10 LEVELS THAN NON (n) CD-DC

Cross-dressing of host DC has been associated recently with allograft rejection.^{29, 30} However, our data now show that DC cross-dressing also occurs during liver allograft tolerance. To further understand the mechanisms involved, we examined the kinetics of CD-DC in the liver grafts and their expression of immune stimulatory/inhibitory molecules. Both the absolute number of DC in the graft and the percentage of DC within the graft hematopoietic cell population peaked on POD 7, then decreased over time, -a pattern similar to that of CD-DC (Fig. 3A). Importantly, > 50% of graft DC were cross-dressed on POD 7 and approximately 40% on POD 14, with 20% still evident on POD 300. Expression of PD-L1 was 12 times higher on CD-DC than on non (n) CD-DC on POD 7, and 3.9 times higher on CD-DC than on DC from control livers (Fig. 3B). These differences were maintained on POD 14 and 30. Co-stimulatory molecules (CD80, CD86) were expressed at similar levels (MFI) to DC from control livers, while nCD-DC showed significantly lower CD80 and CD86 expression levels than on CD-DC on POD 7 (Fig. 3B). Interestingly, although the number of CD-DC in the spleen was much less than graft CD-DC, a small percentage (1–2%) of host spleen DC was cross-dressed as long as POD 300 and expressed high PD-L1 (Suppl Fig. 1). Intracellular cytokine staining revealed that CD-DC in the graft on POD 7 comprised much higher incidences of IL-10⁺ and IL-6⁺ cells than control naïve liver DC or nCD-DC. CD-DC showed a similar low level of IL-12p40⁺ cells to control naïve liver DC, but a higher incidence than nCD-DC (Fig. 3C and D). Taken together, although IL-6 production by CD-DC was also high, graft-infiltrating CD-DC expressed higher levels of PD-L1 and IL-10 than nCD-DC, suggesting a regulatory function of these cells.

CD-DC EXPRESS PD-L1 UNIFORMLY ON THEIR CELL SURFACE

Next, we addressed whether PD-L1 on recipient CD-DC was transferred from donor cells by ascertaining whether PD-L1 co-localized with donor MHC class I molecules on CD-DC on POD 7 (Fig. 4). Both confocal microscopy (Fig. 4A) and flow imaging (Fig. 4B) demonstrated that, in addition to inherent cell surface recipient H-2D^k expression, CD-DC exhibited acquired donor H-2K^b to varying degrees, with most CD-DC showing punctate donor MHC class I expression on their cell surface. Interestingly, PD-L1 was expressed

uniformly on the cell surface of CD-DC, suggesting that the elevated PD-L1 expression observed on these cells was not due to transfer of PD-L1 from donor cells.

LIVER GRAFT CD-DC MARKEDLY SUPPRESS DONOR-REACTIVE HOST T CELL PROLIFERATION

We then investigated the allostimulatory function of the CD-DC. CD-DC and nCD-DC were flow-sorted from host (C3H) spleen and liver grafts (POD 7) and their ability to directly stimulate naïve allogenic T cells (B6) assessed (Fig. 5A and B). As shown in Fig. 5B and C, CD-DC from both host spleens and liver grafts were impaired in their ability to directly activate/induce proliferation of naïve CD4 and CD8 T cells, compared to nCD-DC and splenic DC from naïve C3H mice. Both liver CD-DC (especially) and nCD-DC showed inferior ability to stimulate naïve T cells compared to splenic DC from naïve mice. We also found that host spleen and liver graft CD-DC, that expressed donor MHC on their surface, failed to prime naïve C3H T cells in vitro (semi-direct allo-recognition) (Suppl. Fig. 2).

We also assessed the ex vivo suppressive function of DC from liver transplant recipients. CD-DC and nCD-DC sorted from the graft or spleen on POD 7 were added to naïve C3H responder T cells and allogeneic stimulators (DC from naïve B6 spleen). Both CD-DC and nCD-DC from the graft significantly suppressed the proliferation of CD4 T cells (Fig. 5D and E), whereas splenic DC (both CD- and nCD-DC) showed minimal suppressor function. In addition, only liver graft CD-DC significantly suppressed the proliferation of naïve CD8 T cells (Fig. 5D and E). Notably, the presence of graft CD-DC significantly increased the death of both CD4 and CD8 T cells (Fig. 5D and E) in both proliferating and non-proliferating populations (Fig. 5D). Overall, these data demonstrate both impairment of recipient CD-DC T cell priming ability and a suppressor function that is associated with T cell death.

LIVER GRAFT-INFILTRATING T CELLS EXHIBIT AN EXHAUSTED PHENOTYPE

Given the suppressive function of host DC after liver transplantation, we assessed the phenotype of T cells in the spleen and graft at various times post-transplant. By POD 7, nearly all of the intra-graft T cells exhibited an activated effector/memory phenotype (i.e. CD44^{hi} CD62L^{lo}), that persisted up to 300 days post-transplant (Fig. 6A and B). Importantly, a significant fraction of the CD4 T cells and nearly all of the CD8 T cells infiltrating the grafts on POD 7 exhibited an exhausted phenotype (PD-1 [programed cell death protein 1]⁺, TIM-3[T cell Ig and mucin domain containing-3]⁺)³⁷ (Fig. 6C and D). A concomitant increase in effector/memory T cell differentiation in host spleens was also observed (Fig. 6A and B), but a much lower fraction of splenic T cells demonstrated an exhausted phenotype compared to graft T cells (Fig. 6C and D). Nevertheless, a significantly higher level of CD8⁺ T cells (almost 15–20% of PD-1⁺ cells) in host spleens were exhausted compared to that in naïve spleen.

Interestingly, a lower incidence of CD8⁺ T cells in the graft on POD7 produced IL-2, TNFα (Fig. 6E and F) and IL-6 (data not shown) than those in normal liver, but the former were predominantly IFNγ producers (Fig. 6E and F), suggesting that the graft CD8⁺ T cells were partially exhausted³⁸ and undergoing functional impairment. Taken together, these data

suggest that while some T cells, particularly CD8, become exhausted in the spleen, most T cells become exhausted once they migrate to the liver allograft. In support of this, when graft-infiltrating T cells were stimulated with donor DC ex vivo, they failed to proliferate and died (Suppl Fig. 3).

LIVER GRAFT CD-DC EXHIBIT DIFFERENT GENE EXPRESSION PROFILES COMPARED WITH nCD-DC

Given the differences in co-regulatory molecule expression and suppressor function between CD-DC and nCD-DC isolated from liver grafts, we next assessed their gene expression profiles. RNA from sort-purified freshly-isolated liver graft CD-DC and nCD-DC (POD 7) was analyzed using a Nanostring Mouse Immunology Panel. In the heat map generated using nSolver software, approx 86% of genes showed clear cluster segregation (Fig. 7A). Notably, PD-L1 gene expression was clearly up-regulated in liver graft CD-DC compared with nCD-DC. CD80 and CD86 gene expression was up-regulated in CD-DC compared with nCD-DC, also consistent with cell surface protein expression (Fig. 3). Interestingly, both DAP12, that we have shown negatively regulates liver DC stimulatory function,^{26, 27} and triggering receptor expressed on myeloid cells 2 (TREM2) (a key DAP12-associated cell surface membrane receptor)³⁹ were up-regulated in CD-DC compared to nCD-DC, suggesting their regulatory function^{40, 41}. Since the TREM2-DAP12 pathway in DC is associated mainly with inhibitory signals,⁴¹ this is consistent with our finding that liver graft CD-DC are more regulatory than nCD-DC.

Canonical pathways that were either activated or inactivated in liver graft CD-DC in comparison with nCD-DC were analyzed using Ingenuity Pathway Analysis (IPA) software (Fig. 7B). In the top 10 most significant canonical pathways, “dendritic cell maturation”, “TREM-DAP12 signaling” and “Toll-like receptor signaling” were activated in CD-DC, suggesting that these cells were more mature than nCD-DC. Conversely, pathways inactivated in liver graft CD-DC were related mainly to T cell activation, consistent with CD-DC being less stimulatory compared to nCD-DC. Overall, gene expression analysis showed that liver graft CD-DC and nCD-DC were differentially activated and that liver graft CD-DC were characterized by regulatory signaling pathways to a greater extent than graft nCD-DC.

GRAFT-INFILTRATING DC IN REJECTING (DAP12^{-/-}) LIVER ALLOGRAFTS EXHIBIT FEWER CD-DC THAT EXPRESS LESS DONOR MHC CLASS I AND PD-L1 THAN THOSE IN TOLERATED WT GRAFTS

To determine whether cross-dressing of recipient DC could be observed in rejecting liver allografts. WT (tolerated) or DAP12^{-/-} livers that are rejected acutely²⁷ were transplanted to C3H recipients. We have shown previously²⁷ that DAP12^{-/-} donor livers elicit increased graft T cell infiltration and proinflammatory cytokine expression (IFN γ , TNF α , IL-2, IL-6), consistent with abrogation of transplant tolerance. When we examined rejecting DAP12^{-/-} grafts on POD 7 we found that they had significantly less host CD-DC than tolerated WT grafts (POD 7; Fig. 8A). Furthermore, expression of donor MHC class I (H-2K^b) was reduced significantly on total liver DC and on CD-DC isolated from DAP12^{-/-} grafts on POD 7 (Fig. 8B and C). Moreover, the CD-DC in DAP12^{-/-} grafts expressed significantly

lower levels of PD-L1 than those in WT grafts (Fig. 8D). The total number of host DC infiltrating the graft was also decreased in this model (data not shown). Since we have shown⁴² that high PD-L1/CD86 ratios on circulating plasmacytoid DC correlate with transplant tolerance in human liver transplantation, it is of interest that PD-L1/CD86 ratios on DC infiltrating DAP12^{-/-} liver grafts were significantly lower than on those infiltrating WT liver grafts (Fig. 8E).

Discussion

In this study, we focused on the role of host and in particular, graft-infiltrating recipient DC in liver transplant tolerance. We found large numbers of recipient-derived CD-DC within the allografts during the first few days post-transplant. These CD-DC were PD-L1^{hi}, were less allostimulatory than nCD-DC, suppressed anti-donor host T cell proliferation and promoted the death of alloreactive T cells. The phenomenon of plasma membrane fragment transfer between leukocytes has been reported since 1999⁴³ and called “trogocytosis”, “nibbling” or “cross-dressing.” It has been postulated that molecules acquired by acceptor APC during this process influence the outcome of the subsequent T cell response. Recently, several publications^{29–31} have drawn attention to an important role of CD-DC in the rejection of experimental heart, kidney and skin transplants. However, here we reveal that, conversely, CD-DC in liver allografts that are accepted indefinitely without use of immunosuppressive agents, suppress alloreactive T cell responses rather than promote graft rejection.

Liver allografts are distinct from other types of organ transplants in several other regards. Thus, in addition to the liver’s hematopoietic and regenerative capacity, liver mesenchymal cells produce/release relatively large amounts of donor intact MHC class I molecules⁴⁴ that may be acquired by host-derived DC infiltrating liver allografts via cross-dressing. Indeed, most liver cell types, including hepatocytes, cholangiocytes, LSEC, stellate cells and immune cell populations produce extracellular vesicles that express MHC gene products under in vitro conditions.⁴⁵ Moreover, liver allografts are also enriched in PD-L1^{33, 46} that may confer high PD-L1 expression on graft-infiltrating host DC. By analogy, in cancer, it is evident that tumor-associated PD-L1, that promotes T cell apoptosis, impairs DC-mediated anti-tumor immunity.⁴⁷

When we examined cross-dressed cells by flow imaging and confocal microscopy, we found that CD-DC uniformly expressed recipient MHC class I molecules on the cell surface, but only expressed donor MHC class I in localized spots, indicating that these cells were DC of recipient origin and cross-dressed with donor MHC molecules. Importantly, PD-L1 was expressed uniformly on the cell surface of CD-DC, consistent with its upregulation at the mRNA level. However, the mechanism by which CD-DC expressed relatively high amounts of PD-L1 mRNA and protein remains unknown. One possibility is that, regardless of cross-dressing, these DC are activated by various cytokines to gain the ability to acquire donor MHC molecules and that acquisition of intact donor MHC class I molecules may alter the phenotype and function of the graft-infiltrating DC.

The cell co-culture experiments that we conducted to ascertain which hepatic donor cell population(s) served as the source of MHC class I for host CD-DC revealed that hepatocytes

were the principal source, while only very low levels of DC were cross-dressed with donor MHC class I from LSEC or stellate cells. Marked reduction in the incidence of DC that expressed hepatocyte-derived donor MHC class I when transwells were present, indicated that physical contact was required for cross-dressing. This finding may reflect a lack of migration of extracellular vesicles from the donor hepatocytes through the transwell, as described recently²⁹ in relation to MHC transfer between allogeneic donor and acceptor DC populations.

An important finding in this study is that CD-DC that infiltrate liver allografts exhibit little T cell allostimulatory function but can markedly suppress host alloreactive T cell responses. This regulatory function may be a consequence of high PD-L1 expression by the CD-DC. Indeed, we found that a large number of T cells in the liver allografts were effector memory cells, and that most of them expressed high levels of the exhaustion markers PD-1 and TIM-3. These graft-infiltrating T cells exhibited less proliferative capacity in CFSE-MLR, even in the presence of potent allostimulatory APCs, and underwent apoptotic cell death, as we observed previously in tolerated mouse liver allografts.⁴⁸ High IL-10 secretion by liver graft CD-DC also supports their immune regulatory function. Since the level of IL-10 gene expression by CD-DC and nCD-DC was similar, this suggests that these two populations behave differently after stimulation. In the liver graft rejection model (DAP12^{-/-} grafts), both the percentage and absolute number of CD-DC were less than those detected in tolerated liver allografts, and their surface expression of PD-L1 was significantly lower, again suggesting that these graft-infiltrating CD-DC and their expression of PD-L1 may be important in the induction of liver transplant tolerance.

PD-L1 expression is upregulated in liver grafts after transplantation in both mice and humans and the PD-L1-PD-1 pathway has been implicated previously⁴⁹ in regulation of anti-donor immunity. Moreover, Morita et al⁴⁶ have reported that mesenchyme-mediated immune control, in particular PD-L1 expression by the graft, is important for the development of mouse liver transplant tolerance. Thus, they showed that blocking of the PD-1/PD-L1 pathway or transplantation of PD-L1 KO grafts, led to pronounced leukocyte infiltration and abrogation of transplant tolerance. In this study, we focused on intra-graft DC rather than liver mesenchymal cells, because of evidence that liver DC can subvert T cell responses^{13, 24} and promote allograft survival.^{21, 22} Indeed, we found that cell surface PD-L1 expression by CD-DC was many-fold higher than that detected on nCD-DC on POD 7, and that this effect correlated with suppression of host anti-donor T cell proliferation. In a previous report,⁵⁰ activated hepatic stellate cells expressing PD-L1^{hi} were found to suppress activated T cells by promoting their apoptosis, but failed to inhibit/regulate their proliferation. This suggests that liver-resident cells expressing PD-L1^{hi} have a regulatory function *in situ*, but may lack the ability to regulate systemic T cell activation. Considering that after liver transplantation, subsequent skin or heart allografts from the same donor strain are accepted spontaneously,¹ local and systemic T cell regulation appears to occur after liver transplantation; CD-DC, therefore, may regulate anti-donor T cell responses in the periphery or in secondary lymphoid tissue. The data we present in this study (Fig. 6) indicate that, in WT liver allograft recipients, host T cells respond normally to donor alloAg in the spleen, but that once they migrate to the liver, they gain TIM-3/PD1 expression, suggesting that the tolerogenic effect may be localized to the liver.

We propose a new concept to help explain the induction/maintenance of liver transplant tolerance. Thus, graft-infiltrating PD-L1^{hi} host DC may serve to subvert anti-donor T cell responses and promote exhaustion/death of graft-infiltrating CD8⁺ T cells and as a result, promote transplant tolerance. Since, after liver allotransplantation, the peak of CD-DC in the graft is on POD 7 and that of infiltrating T cells (especially CD8⁺ T cells) is on POD 14, the fate of the graft may be determined within the first two weeks of transplantation. Elucidation of mechanisms whereby immunoregulatory CD-DC arise in the graft may provide future directions towards the promotion of transplant tolerance in the clinic.

In conclusion, a large proportion of host-derived CD-DC expressing high levels of PD-L1 and IL-10, and with the capacity to subvert host anti-donor T cell responses, are evident in MHC mismatched mouse liver allografts early post-transplant. These CD-DC may play a key role in the regulation of alloimmunity and in the promotion of liver transplant tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ab	antibody
APC	antigen-presenting cell
CD	cross-dressed
CFSE	carboxyfluorescein succinimidyl ester
DC	dendritic cell
DAP12	DNAX activation protein of 12kDa
EV	extracellular vesicles
FACS	fluorescence-activated cell sorting
IFN-γ	interferon-gamma
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction

mRNA	messenger RNA
NPC	non-parenchymal cell
PD-(L)1	programmed death- (ligand) 1
PMA	phorbol 12-myristate 13-acetate
POD	postoperative day
TEM	T effector memory
TCM	T central memory
TIM-3	T cell Ig and mucin-domain containing-3
TN	T naïve
TREM	triggering receptor expressed on myeloid cells
YFP	yellow fluorescent protein
WT	wild-type

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Author names in bold designate shared co-first authorship

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FIG. 1. Progressive disappearance of donor DC and emergence and persistence of cross-dressed (CD) host DC following mouse liver transplantation

(A) The mouse liver transplantation model used B6 (WT), B6 SJL CD45.1 or B6 CD11c-YFP (MHC class I: H-2K^b) livers transplanted into C3H (CD45.2; MHC class I: H-2D^k) recipients. Graft recipients were euthanized on post-operative days (POD) 1, 3, 7, 14, 30 and 300. Liver non-parenchymal cells (NPC) and splenocytes were isolated and DC analyzed by flow cytometry. (B) Donor (x-axis) and recipient (y-axis) MHC class I expression was analyzed on liver graft and spleen DC defined as CD3⁺, NK1.1/CD49b⁺, B220⁺, CD11b⁺ and CD11c⁺ at the time-points indicated and compared to DC from normal C3H (recipient) and B6 (donor) controls. Data are representative of 3–5 mice per time point. (C) Representative flow plots of liver graft DC after B6 SJL CD45.1 to C3H transplantation. CD45.1 (donor) and CD45.2 (recipient) gating is shown for graft DC and for normal C3H and normal B6 CD45.1 liver DC. Representative data from 3–5 mice are shown for each time-point. (D) Two-photon intravital imaging was performed on control native liver (B6 CD11c-YFP) and liver grafts at various times after B6 CD11c-YFP to C3H transplantation. Donor DC are shown in green and vessels in red. (E) Gating strategy used to analyze cross-dressed (CD) immune cells is shown. Liver NPC were isolated on POD 7 after B6 WT to C3H transplantation and analyzed by flow cytometry. Representative data from n=4 mice are shown. (F) The contribution of each CD immune cell type to the overall CD population.

Bars are means + SEM. n = 4 mice; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. P values were generated by 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

FIG. 2. In vitro transfer of MHC class I molecules from allogeneic liver cells to DC

(A) Bone marrow-derived DC from C3H mice (H-2D^k) were co-cultured with purified hepatocytes, liver sinusoidal endothelial cells (LSEC) or stellate cells from B6 mice (H-2K^b) for 20 h, in the absence or presence of 0.4 μ m pore transwells, and then harvested and analyzed by flow cytometry for co-expression of MHC class I of the donor (H-2K^{b+}) and the acceptor (H-2D^k). (B) Percentages of C3H DC (CD11c⁺ H-2D^{k+}) cross-dressed with B6 H-2K^{b+} molecules (CD-DC) in DC co-cultures, in the presence or absence of trans-wells. * $p < 0.05$ by one-way ANOVA; data are from $n = 3-4$ individual experiments.

FIG. 3. Graft-infiltrating host DC and cross-dressed DC (CD-DC) express high PD-L1 and IL-10

(A) Chronological changes in the absolute number and incidence of total DC and CD-DC in liver allografts determined by flow cytometry at various times pre- (normal control) and post-transplant. $n=3-6$ mice per time point. (B) Expression of co-inhibitory (PD-L1) and co-stimulatory (CD80, CD86) molecules by CD-DC and non (n)CD-DC in liver grafts at various times post-transplant. Representative histograms of these molecules on POD 7 are shown in the column on the left and average mean fluorescence intensity (MFI) + SEM for each molecule on POD 0 (normal control), 7, 14, 30 and 300 is shown on the right. Normal B6 livers served as controls. $n=3-6$ mice per time point. * $P<0.05$; *** $P<0.001$. (C) IL-10, IL-6 and IL-12p40 production by graft DC on POD 7 compared to control normal C3H liver DC. Representative values from $n=3$ transplanted mice are shown for each cytokine. (D) Incidences of DC positive for each cytokine are shown compared to normal C3H control livers. Results (means + SEM) are from $n=3$ graft recipients/group. ** $p<0.01$; *** $p<0.001$.

P values were generated by 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

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FIG. 4. Cross-dressed recipient DC uniformly express PD-L1 on the cell surface

(A) Confocal microscopy of flow-sorted, cross-dressed (CD)-DC isolated from liver grafts on POD 7. The Ab used and corresponding color are indicated at the top of each column (B–C). Flow imaging of graft-infiltrating host (C3H; H-2D^k) CD-DC on POD 7. (B) DC were stained for CD11c, H-2K^b (donor) and H-2D^k (recipient) and 2 representative images are shown, with normal B6 (donor) and C3H (recipient) liver DC as controls. (C) DC were stained for CD11c, H-2K^b (donor), H-2D^k (host) and PD-L1. Co-localization of PD-L1 and MHC class I (Merge 1) was also examined. Two representative profiles from n=10 are shown.

FIG. 5. Liver graft cross-dressed (CD)-DC fail to stimulate naive donor T cells and markedly suppress donor-reactive host T cell proliferation

(A) Protocol for assessing CD-DC and non (n)CD-DC stimulatory and regulatory function in CFSE-MLR. B6 livers were transplanted into C3H recipients that were euthanized on POD 7. CD11c⁺ cells were isolated from the grafts and spleens by positive selection (MACS). After staining for donor MHC class I (H-2K^b), H-2K^b⁺ cells (CD-DC) and H-2K^b⁻ cells (nCD-DC) were sorted by FACS. CFSE-MLR were performed at a DC to T cell ratio of 1 : 10. (B) Assessment of the stimulatory potential of graft-infiltrating recipient DC: spleen (Sp) or liver graft CD-DC or nCD-DC (stimulators) isolated on POD 7 were co-cultured with allogeneic B6 Sp T cells (responders). Negative control: no stimulators; positive control: C3H Sp DC stimulators. Cells were harvested after 5 d culture. Flow cytometry data are representative of n=3 independent experiments. (C) Percentages of proliferating CD4⁺ and CD8⁺ T cells are shown (means + SEM). Data were obtained from n = 3 independent experiments. ***P* < 0.01. (D) Assessment of the regulatory potential of graft-infiltrating recipient DC: spleen or liver graft CD-DC or nCD-DC (putative regulators) were isolated on

POD 7 and co-cultured with C3H (host) spleen T cells (responders) and B6 (donor) spleen DC (stimulators) (T cells : DC : (n)CD-DC = 10:1:1). Data shown are representative of $n = 3$ independent experiments. The top 2 panels show % divided cells within live $CD4^{+}$ or $CD8^{+}$ T cells; the lower 2 panels show % dead cells (Zombie⁺) within $CD4^{+}$ or $CD8^{+}$ T cells. **(E)** Percentages of proliferating cells (upper panels) and dead cells (lower panels) from D are shown (means + SEM). Data were obtained from $n = 3$ independent experiments. * $P < 0.05$; ** $P < 0.01$. P values were generated by 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

FIG. 6. Liver graft-infiltrating T cells exhibit an exhausted phenotype

C3H recipients of B6 livers were euthanized on POD 7, 14, 28, and 300 and T cell populations in the graft and spleen analyzed by flow cytometry. **(A)** Upper row; absolute number and percentage of CD4⁺ and CD8⁺ T cells for each time-point post-transplant. Lower row; effector/memory T cells (T_{EM}) were also enumerated. Data are means ± SEM (symbols without error bars indicate very small SEM); n = 3–6 mice per time-point in each group. **(B)** Naïve and memory T cell subsets were defined based on their differential expression of CD62L and CD44; i.e. naïve T cells (T_N): CD62L⁺ CD44⁻, central memory T cells (T_{CM}): CD62L⁺ CD44⁺, and effector/memory T cells (T_{EM}): CD62L⁻ CD44⁺. Graft-infiltrating and splenic T cells were determined on POD 0 (control: normal mice) and on POD 7 and 300. Representative flow data are shown; n = 3–6 mice per time point in each group. **(C)** Expression of PD-1 and TIM-3 by CD4⁺ and CD8⁺ cells in liver grafts and spleens determined by flow cytometry on 7 POD. Representative flow data for PD-1 and TIM-3 double positive cells are shown. Normal C3H mouse livers served as controls. **(D)** Incidence of PD-1 and TIM-3 double positive (i.e. exhausted) CD4⁺ and CD8⁺ cells. Data

are means + SEM; n = 3 mice per group. * $P < 0.05$; **** $P < 0.0001$. **(E)** Representative flow data showing cytokine (IFN γ , TNF α , IL-2) expression by graft-infiltrating T cells (CD4⁺ and CD8⁺) on POD 7 compared to normal C3H control T cells. n = 3 mice per group. **(F)** Compilation of data from E. Results are means + SEM percent positive cells; n = 3 mice per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. P values were generated by 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.

FIG. 7. Liver graft cross-dressed (CD)-DC exhibit a gene expression profile distinct from non (n)CD-DC and related to distinct canonical pathways

(A) Liver graft CD-DC and nCD-DC were isolated on POD 7 and gene expression compared using the Nanostring Mouse Immunology Panel. The heatmap shows segregation of gene expression in each DC type. Red indicates an increase in expression and green indicates a decrease in gene expression relative to the other DC type. (B) Ingenuity Pathway Analysis (IPA) was used to compare canonical pathways between liver graft CD-DC and nCD-DC. Three analyses are depicted. First, the color of the bar indicates predicted pathway activation (orange) or predicted inhibition (blue) in CD-DC compared with nCD-DC, according to the z-score, which is used to compare the dataset with the canonical pathway patterns mathematically. White bars are those with a z-score at or very close to 0. Second, the width of each bar denotes the -log of the p-value which is calculated by Fisher's exact test right-tailed and shown at the top of the x-axis. Thus wider bars equate to increased significance. Third, the interconnected orange points represent the ratio that is calculated as follows: number of genes in a given pathway divided by the total number of genes that make up that pathway based on the previous literature.

FIG. 8. Rejecting liver allografts from DAP12^{-/-} donors exhibit less cross-dressed (CD)-DC that express lower levels of donor MHC class I and PD-L1

Liver transplantation was performed using B6 DAP12^{-/-} donors (rejection model) compared with WT B6 donors (tolerance model). (A) Absolute number and incidence of graft CD-DC at various times post-transplant. CD-DC in DAP12^{-/-} grafts were significantly lower ($27 \pm 4\%$) on POD 7 compared with WT grafts ($60 \pm 8\%$); $n = 3-5$ mice per time point in each group. * $P < 0.05$. (B) Expression of donor MHC class I (H-2K^b) (MFI) on WT and DAP12^{-/-} liver graft total DC (left) and CD-DC (right) on POD 7 and 14. (C) Expression of co-inhibitory and co-stimulatory molecules (mean fluorescence intensity; MFI) by CD-DC and non (n)CD-DC in DAP12^{-/-} or WT grafts determined by flow cytometry on POD 7; $n =$

3–5 mice per time point in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **(D)** Ratio of PD-L1 to CD86 expression by liver graft DC on POD 7 determined on the basis of mean fluorescence intensity (MFI). There were $n = 3$ –5 mice in each group and data are means + SEM. * $p < 0.05$. P values were generated by 1-way ANOVA followed by Tukey-Kramer multiple comparisons test.