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Selective Expansion of Allogeneic Regulatory T Cells by Hepatic Stellate Cells: Role of Endotoxin and Implications for Allograft Tolerance

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Hepatic stellate cells (HSCs) may play an important role in hepatic immune regulation by producing numerous cytokines/chemokines and expressing Ag-presenting and T cell coregulatory molecules. Due to disruption of the endothelial barrier during cold-ischemic storage and reperfusion of liver grafts, HSCs can interact directly with cells of the immune system. Endotoxin (LPS), levels of which increase in liver diseases and transplantation, stimulates the synthesis of many mediators by HSCs. We hypothesized that LPS-stimulated HSCs might promote hepatic tolerogenicity by influencing naturally occurring immunosuppressive CD4+ CD25+Foxp3+ regulatory T cells (Tregs). Following their portal venous infusion, allogeneic CD4+ T cells, including Tregs, were found closely associated with HSCs, and this association increased in LPS-treated livers. In vitro, both unstimulated and LPS-stimulated HSCs upregulated Fas (CD95) expression on conventional CD4+ T cells and induced their apoptosis in a Fas/Fas ligand-dependent manner. By contrast, HSCs induced Treg proliferation, which required cell–cell contact and was MHC class II-dependent. This effect was augmented when HSCs were pretreated with LPS. LPS increased the expression of MHC class II, CD80, and CD86 and stimulated the production of IL-10 and TNF-α by HSCs. Interestingly, production of IL-1α, IL-1β, IL-6, IL-10 and TNF-α by HSCs was strongly inhibited, but that of IL-10 enhanced in LPS-pretreated HSC/Treg cocultures. Adipotively transferred allogeneic HSCs migrated to the secondary lymphoid tissues and induced Treg expansion in lymph nodes. These data implicate endotoxin-stimulated HSCs as important immune regulators in liver transplantation by inducing selective expansion of tolerance-promoting Tregs and reducing inflammation and alloimmunity. The Journal of Immunology, 2012, 188: 3667–3677.

The liver receives 75–80% of its blood supply from the portal vein that conveys food-derived and microbial products, Ags, endotoxins, and xenobiotics and plays a major defense role against potentially harmful factors on a continuous basis. This function is performed by the various hepatic cell populations in a coordinated manner. Despite its role of immunologically active cell types (1, 2), the liver demonstrates a remarkable capacity to impart immune tolerance, as evidenced by viral persistence (e.g., hepatitis B and C), parasite infection (e.g., malaria parasite), and tumor metastasis. Liver tolerance is exemplified by the spontaneous acceptance of hepatic allografts across MHC barriers in animal models (3, 4) and human liver transplants (5, 6). Specific interactions between hepatic cell types and those of the immune system may determine the short- and long-term fate of liver allografts. Thus, donor liver-derived passenger leukocytes (microchimerism) can subvert antidonor T cell responses (7–9). Other liver nonparenchymal cells can also exhibit tolerogenic properties (2). Thus, although hepatic dendritic cells (DCs), Kupffer cells (KC), and liver sinusoidal endothelial cells (LSECs) have been studied extensively in this regard (10, 11), many unanswered questions remain concerning mechanisms underlying the tolerogenic properties of liver nonparenchymal cells.

Hepatic stellate cells (HSCs), which are located in the perisinusoidal space (Space of Disse), have been investigated extensively for their role in liver fibrosis (12–14). Under physiological conditions, HSCs are quiescent and store most of the body’s retinoids (~80%). During liver injury, they undergo activation characterized by the loss of retinoids and transdifferentiation into highly proliferative and fibrogenic myofibroblast-like cells. The role of HSCs in hepatic immunobiology, and in particular transplant immunology, is understudied, despite their ability to produce an array of cytokines/chemokines (14–16) and their expression of Ag-presenting and coregulatory molecules (17–21). Fully activated, IFN-γ–stimulated HSCs can inhibit allogeneic effector
**ROLE OF LPS-STIMULATED HSCs IN Treg EXPANSION**

Cultured HSCs incubated in the absence or presence of 100 ng/ml LPS for 24 h were harvested using trypsin/EDTA, suspended in DMEM, washed with PBS (2% BSA), and stained with the following Abs: MHC class I, MHC class II, CD40, CD80, CD86, ICAM-1, VCAM, B7-H1, and FasL (BioLegend). The cells were then fixed in 4% paraformaldehyde on ice for 20 min, permeabilized with 0.1% saponin for 30 min at room temperature, and stained with rabbit polyclonal anti-desmin Ab (Abcam, Cambridge, MA) at 1:200 dilution, followed by goat anti-rabbit IgG (H+L)-Pacific Blue secondary Ab (Invitrogen-Molecular Probes, Eugene, OR) at 1:400 dilution. In some experiments, expression of intracellular cytokines IL-6, IL-10, or Tnf-α was also determined. The stained cells were detected on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.1 (Tree Star, Ashland, OR).

**T cell isolation and purification**

A single-cell suspension of splenocytes of BALB/c mice was treated with 0.84% NH₄Cl to lyse RBCs, and then T cells were enriched using a sterile nylon wool fiber column according to the manufacturer’s protocol (Wako, Osaka, Japan). T lymphocytes were stained for CD3, CD4, CD8, CD25 (BioLegend), CD127 (eBioscience, San Diego, CA), and 7-amino- actinomycin D (7-AAD; BD Pharmingen). Stained cells were acquired using a BD FACSaria (BD Biosciences). Dead cells were stained with 7-AAD for exclusion, and the viable CD3⁺CD8⁻ cells with the following compositions were sorted: 1) bulk CD4⁺ T cells (CD4⁺CD25⁻ including Tregs); 2) conventional CD4⁺ T cells (CD4⁺CD25⁻ only); and 3) natural Tregs (CD4⁻CD25⁺CD127⁻/low) (31). Following sorting, individual cell populations were tested for their purity, which was always >95%. Nearly 95% or more of the sorted Tregs were found to be positive for Foxp3 (data not shown).

**Immunofluorescence microscopy**

HSCs seeded on coverslips in coculture with Tregs or liver sections (placed on glass slides) were stained with rabbit anti-desmin (Abcam), rat anti-Foxp3 (Alexis), rabbit anti-Ki67 (Abcam), rat anti-CD4, or rat anti-mouse CD31 Abs (BioLegend). Following primary staining, goat anti-rabbit Alexa Fluor 488, goat anti-rat Alexa Fluor 594, or donkey anti-mouse Alexa Fluor 594, or donkey anti-rabbit Alexa Fluor 488, goat anti-rat Alexa Fluor 594 or donkey anti-mouse Alexa Fluor 594 Abs were used as secondary Abs, whereas DAPI was used as a nuclear stain. Coverslips or sections were mounted using gelvatol. The slides were placed at 4°C overnight before microscopic examination (25).

**Coculture of HSCs with allogeneic CD4⁺ T cells**

Overnight-cultured HSCs were treated with 270 μM GdCl₃ (Sigma-Aldrich) for 24 h to block the activity of contaminating KCs, if any, and then washed and stimulated with LPS (100 ng/ml) for 24 h. The cells were then washed and cocultured in fresh medium with CFSE-labeled bulk CD4⁺ T cells, conventional CD4⁺ T cells, or Tregs (at 1:10 ratio in 96-well flat-bottom plates). CFSE labeling was carried out as described previously (32). Polyomyxin B (300 ng/ml; Sigma-Aldrich) was added to the cultures to block the direct effect of any residual LPS on CD4⁺ T cells. In some experiments, HSCs were cultured in inserts (0.45-m pore size) and separated from Tregs placed in the bottom portion of the wells (BD Falcon) to determine whether their influence on T cells was due to soluble mediators or required cell–cell contact. Apoptosis was assessed by staining with 7-AAD and Annexin-V, whereas cell proliferation was measured by CFSE dilution assay (32). In some experiments, blocking Abs (anti-FasL, anti-Fas, anti-I-A/I-E) or the costimulation blocking agent CTLA4-Ig were added at the start of cultures.

**Quantification of cytokines**

Cell-free supernatants were collected to measure concentrations of cytokines using a mouse 20-plex LumineX beadset (Biosource International, San Diego, CA) and LumineX 100 IS apparatus (LumineX, Austin, TX).

**Determination of the suppressive function of HSC-expanded Tregs**

Untreated or LPS-pretreated B6 HSCs were cocultured with purified BALB/c Tregs for 5 d in the presence of 200 U/ml IL-2, as described above. Tregs were recovered and, to increase their yield, reincubated with fresh B6 HSCs plus IL-2 for an additional 5 d. Tregs recovered from culture were characterized for their expression of GITR, programmed death-1 (PD-1), LAG-3, CD103, CCR7, CD62L, and intracellular CTLA4 and IL-10. To determine the suppressive potential of HSC-expanded Tregs, B6-Tregs (Ly5.2) were expanded with BALB/c HSCs and then added at ratios of 1:10 and 1:20 in coculture of CFSE-labeled conventional B6-CD4⁺ T cells (Ly5.2) and
γ-irradiated BALB/c splenocytes (as stimulators) in 96-well plates for 96 h (32). To some cultures, no Tregs or naïve or anti-CD3/CD28 Ab-activated conventional B6 CD4+ T cells (isolated from Ly5.1/Ly5.2 hybrid mice) were added to serve as negative controls. Suppression was determined by flow cytometry.

Adoptive transfer of Tregs

BALB/c HSC-expanded B6 Tregs were labeled with CFSE and adoptively transferred to B6 mice (1 × 10^6 Tregs/animal). One week later, the animals were euthanized, cells isolated from spleens and lymph nodes (axillary, inguinal, mesenteric, and renal), and the level of expression of Foxp3 in the CFSE-labeled cells assessed by FACS to determine their stability in vivo. Adoptive transfer of HSCs

B6 HSCs (untreated or LPS-pretreated) were adoptively transferred to BALB/c mice through the lateral tail vein (5 × 10^6 cells/mouse). After 5 d, cells from blood, spleens, and lymph nodes were isolated and assessed for total number of the natural Tregs (CD4+Foxp3+Helios+). In some experiments, GFP+B6 HSCs were adoptively transferred to BALB/c mice, and on day 2 and 5 after cell transfer, spleens and lymph nodes were collected, cryopreserved, and the migration of allogeneic HSCs and their interaction with Tregs were examined by immunohistology.

Statistical analysis

Results are expressed as means ± SD. Statistical significance between groups was determined by Student t test using the Prism 5 software package (GraphPad, San Diego, CA). A p value <0.05 was considered statistically significant.

Results

**LPS modulates the expression of Ag-presenting and T cell coregulatory molecules on HSCs**

HSCs expressed relatively high levels of surface MHC class I, but their expression of MHC class II as well as T cell costimulatory molecules (CD40, CD80, and CD86) was very low (Fig. 1). LPS significantly increased expression of these molecules, except MHC class I. HSCs also expressed ICAM-1, VCAM, programmed death ligand-1 (B7-H1), and FasL (CD95L). LPS significantly increased surface expression of VCAM and FasL, as well as the intracellular expression of IL-10 in HSCs (Fig. 1).

**Retention of allogeneic CD4+ T cells by the liver**

Despite their higher frequency in the liver (33), hepatic CD4+ T cells (30–40% of total intrahepatic lymphocytes in mice) have been understudied compared with CD8+ T cells (34). In this study, we first determined whether HSCs might display enhanced interactions with allogeneic (BALB/c) CD4+ T cells following cold I/R of the B6 mouse liver. Consistent with previous observations (24, 25), LSEC-specific CD31 staining was markedly reduced in the cold I/R livers, indicating disruption of the endothelial barrier (Fig. 2A). Numerous infused allogeneic CD4+ T cells were retained in the liver, and I/R did not increase their retention (Fig. 2B). However, LPS pre-exposed livers (both unprepared or normal and cold-preserved) retained significantly greater numbers of CD4+ T cells than the untreated livers (Fig. 2B, 2E); only a few CD4+ cells were observed in the control livers that were repurposed without allogeneic cells (Fig. 2C). In contrast to the allogeneic cells, significantly less retention of the infused syngeneic CD4+ T cells was observed in the cold I/R livers (Fig. 2D, 2E). These results demonstrate that the CD4+ T cells observed in the livers (Fig. 2B) were predominantly allogeneic. By high-power imaging, allogeneic CD4+ T cells were found to be retained mainly in the sinusoids of control livers (data not shown), and in close proximity to HSCs in cold-preserved livers (Fig. 2F, 2G).

Interestingly, Foxp3+ Tregs were also found in close association with HSCs in cold I/R livers, and this interaction was enhanced upon LPS pretreatment (Fig. 2H, 2I, 2L). In fact, ~50% of the retained Tregs were closely associated with HSCs. Immuno-staining of HSC/Treg cocultures also demonstrated the adherence of greater number of Tregs to LPS-pretreated (Fig. 2K) compared with untreated HSCs (Fig. 2J). These data indicate the ability of the liver to retain allogeneic CD4+ T cells regardless of cold preservation with augmentation of the effect upon LPS pretreatment (35). The data further suggest that HSCs may play an important role in this phenomenon, especially when the endothelial barrier is disrupted due to cold I/R.

**HSCs promote apoptosis of conventional CD4+ T cells via Fas–FasL interaction**

Fas–FasL and B7-H1–PD-L1 interactions promote T cell death (36, 37). We next considered whether control or LPS-stimulated HSCs might induce conventional CD4+ T cell death via interactions of FasL and B7-H1 on HSCs with Fas and PD-1, respectively, on T cells. As shown in Fig. 1, HSCs displayed low levels of FasL and B7-H1, and LPS treatment significantly increased FasL but not B7-H1 expression. Interestingly, Fas ex-
expression increased strikingly on conventional CD4+ T cells cocultured with HSCs, regardless of LPS pretreatment (Fig. 3A). However, surface expression of PD-1 on these T cells was not detected, without or with coculture with HSCs ± LPS (Fig. 3B). Because PD-1 is expressed on peripheral CD4+ and CD8+ T cells, NKT cells, B cells, monocytes, and some DC subsets upon their activation by cytokines such as IL-2, IL-7, IL-15, and IL-21 (38, 39), our data suggest that HSCs do not activate CD4+ T cells or produce cytokines that induce their expression of PD-1. Furthermore, HSCs did not upregulate the expression of the early activation markers CD69 or CD71 on conventional CD4+ T cells (data not shown). CFSE dilution assay also confirmed that HSCs did not induce proliferation of conventional CD4+ T cells (Fig. 3C). In coculture with untreated or LPS-pretreated HSCs, apoptosis of conventional CD4+ T cells increased significantly and similarly, as indicated by 7-AAD and Annexin V staining (Fig. 3D, upper panel and bar graph). Partial reversal of HSCs ± LPS-induced apoptosis of conventional CD4+ T cells occurred in the presence of anti-FasL–blocking Ab (Fig. 3D, lower panel and bar graph). Apoptosis of conventional CD4+ T cells induced by coculture with
HSCs ± LPS was not altered by B7-H1–blocking Ab (data not shown). These results suggest that HSCs induce apoptosis of conventional CD4⁺ T cells primarily via a Fas-FasL–dependent mechanism.

**LPS-stimulated HSCs induce expansion of allogeneic Foxp3⁺ Tregs**

In all Treg experiments, exogenous IL-2 (200 U/ml) was added because it is necessary for Treg survival, and our preliminary experiments showed no expression of IL-2 by unstimulated or LPS-stimulated (100 ng/ml) HSCs. Bar graph shows the percentage of CD4⁺ T cells undergoing apoptosis (7-AAD⁺ Annexin V⁻, 7-AAD⁺/Annexin V⁺, and 7-AAD⁻ Annexin V⁺ cells) in the absence or presence of anti-FasL blocking Ab. The Ab was added to the HSC culture at a concentration of 10 μg/ml, 1 h prior to the start of coculture with CD4⁺ T cells. Incubation of bulk allogeneic CD4⁺ T cells with untreated HSCs increased the numbers of the Foxp3⁺ Tregs significantly compared with controls; this effect was enhanced in coculture with LPS-pretreated HSCs (Fig. 4A and bar graph). These Treg populations were positive for Helios, a member of the Ikaros transcription factor family. Helios is preferentially expressed by naturally occurring Tregs, but not by induced Tregs (40) (Fig. 4A). CFSE dilution assay showed proliferation of ~60 and 75% of Helios⁺ Tregs in the presence of control and LPS-pretreated HSCs, respectively (Fig. 4B). These data indicate that LPS-stimulated HSCs induce the expansion of Tregs from the bulk CD4⁺ T cell population. The HSC-induced Treg expansion appeared to be alloantigen-specific because HSCs failed to expand syngeneic Tregs (data not shown).
We next examined the influence of HSCs on purified Tregs. CFSE-labeled allogeneic Tregs were cultured alone or with HSCs (untreated or LPS-pretreated). Significant expansion of purified Tregs occurred in coculture with untreated HSCs, and this effect was augmented when HSCs were pretreated with LPS (Fig. 5A). In a parallel experiment, separation of HSCs from Tregs in transwell cultures prevented Treg expansion (Fig. 5A and bar graph), indicating the need for cell–cell contact.

**HSC-induced proliferation of Tregs is dependent on MHC class II**

Even though HSCs produce a number of mediators, including TGF-β and retinoic acid, that might affect the activation/function of Tregs (41), the data shown in Fig. 5A demonstrated that HSC-induced Treg expansion was contact dependent. Furthermore, incubation of Tregs in medium conditioned by HSCs (± LPS) did not stimulate their expansion (data not shown). Key cell surface-expressed molecules, such as MHC class II and CD80/CD86, have been implicated in Treg proliferation (42–44). LPS increased the expression of MHC class II, CD80, and CD86 on HSCs significantly (Fig. 1). Upon examining the role of these upregulated molecules, blockade of MHC class II, but not the costimulatory molecules, significantly prevented HSC-induced Treg expansion (Fig. 5B and bar graph), suggesting a predominant role of TCR signaling in this effect.

**Bidirectional interaction between HSCs and Tregs in the production of cytokines**

Cytokines produced in the hepatic microenvironment following transplantation influence the course of allograft acceptance or rejection. Therefore, we ascertained the influence of HSC–Treg interaction on the production of anti-inflammatory (IL-10) and proinflammatory (IL-1α, IL-1β, IL-6, and TNF-α) cytokines. HSCs produced these cytokines spontaneously, and LPS pretreatment increased their production by HSCs significantly (Fig. 5A). Basal production of these cytokines by Tregs was very low/negligible, and, in coculture, their basal release by HSCs was abrogated. Interestingly, production of IL-10 by LPS-pretreated HSCs increased and that of IL-1α, IL-1β, IL-6, and TNF-α was reduced significantly in coculture with Tregs (Fig. 6A). Intracellular staining further confirmed production of these cytokines by HSCs (Fig. 6B). Although we did not observe IL-6 and TNF-α expression by Tregs cultured alone or with HSCs (± LPS) (data not shown), their IL-10 expression increased in coculture with HSCs (Fig. 7A). These data indicate that bidirectional interaction between HSCs and Tregs can markedly affect the balance between pro- and anti-inflammatory cytokine production.

**HSC-expanded Tregs retain regulatory properties and exhibit potent suppressive function**

The principal function of Tregs is maintenance of homeostasis by the suppression of exaggerated immune responses. Therefore, we examined Tregs for expression of molecules that are responsible for suppressive function. About 80–90% of HSC-expanded Tregs maintained Foxp3 expression (data not shown) and were uniformly positive for GITR (Fig. 7A), a potent T cell costimulatory receptor and regulator of Treg function, deficiency of which abrogates the suppressive function of Tregs and increases T cell proliferation (45). HSC-expanded Tregs expressed high levels of PD-1 (surface) and CTLA-4 (intracellular), which are implicated in induction of allograft tolerance by suppressing the activation, proliferation, and survival of CD4+ and CD8+ T cells (37, 46). The proliferating Tregs were positive for LAG-3, a protein expressed by activated natural Tregs and an augmenter of their regulatory function (47), CD103 (integrin αEβ7), a marker for memory/activated Tregs, and CCR7 and CD62L, the molecules implicated in their migration in nonlymphoid tissues (48).

We then tested the suppressive function of Tregs cocultured with HSCs (unstimulated and LPS stimulated). Tregs that proliferated in coculture with untreated or LPS-pretreated HSCs caused greater suppression of alloreactive CD4+ T cell proliferation than freshly isolated Tregs. Only ~20% alloreactive CD4+ T cells proliferated significantly in coculture with untreated or LPS-pretreated HSCs (Fig. 6B). Although we did not observe IL-6 and TNF-α expression by Tregs cultured alone or with HSCs (± LPS) (data not shown), their IL-10 expression increased in coculture with HSCs (Fig. 7A). These data indicate that bidirectional interaction between HSCs and Tregs can markedly affect the balance between pro- and anti-inflammatory cytokine production.

**FIGURE 5.** Proliferation of purified Tregs by HSCs requires cell contact. (A) Tregs were cultured alone or with HSCs (untreated or LPS-pretreated [100 ng/ml]) at an HSC/Treg ratio of 1:10 for 5 d in the presence of 200 U/ml IL-2. CFSE dilution assay was performed to determine the proliferation of Foxp3+ cells. Proliferation of Tregs was blocked when the cells were separated from HSCs using transwell culture inserts. Bar graph shows the absolute numbers of Treg at the end of culture period (n = 3). (B) HSC-induced Treg expansion is mediated by MHC class II on HSCs. HSCs, preincubated with 100 ng/ml LPS for 24 h, were treated with 5 μg/ml anti-MHC class II (purified anti-mouse I-A/E) Ab, 5 μg/ml of the costimulation blocking agent CTLA-4-Ig, or control IgG for 30 min. The medium was aspirated, and CFSE-labeled Tregs were added to the culture wells in fresh RPMI-1640 medium. At the end of incubation (day 5), proliferation of Tregs was measured by CFSE dilution assay. Bar graph represents percent Treg proliferation (mean ± SD) (n = 3). **p < 0.05, ***p < 0.01.
when cultured with Tregs harvested from HSC cocultures (untreated and LPS pretreated, respectively) in contrast to ~35% that proliferated with freshly isolated Tregs at the same suppressor/effector cell ratio (Fig. 7B). In contrast, the addition of naive or activated conventional B6 CD4+ T cells (coexpressing Ly5.1 and Ly5.2) (negative controls) instead of Tregs (at 1:10 ratio) demonstrated very mild inhibition of proliferation (Fig. 7B). These data suggest that in vitro HSC-expanded Tregs retain a suppressive phenotype and function. Upon examining their stability in vivo following adoptive transfer, HSC-expanded Tregs located in the spleen and lymph nodes were found to express a high level of Foxp3 even after a week in comparison with the anti-CD3/CD28 Ab-expanded Tregs (Fig. 7C). These data indicate superior suppressive potential and in vivo stability of HSC-expanded Tregs.

**HSCs preferentially increase Tregs in lymph node in vivo**

To ascertain the in vivo relevance of HSC-induced Treg expansion, we adoptively transferred B6 HSCs (untreated or LPS-pretreated) into BALB/c mice. After 5 d, naturally occurring Tregs (CD4+ Foxp3+Helios+) were assessed in blood, spleen, and lymph nodes. The number of these Tregs in the blood decreased in mice that received HSCs (Fig. 8A, 8B). Although the proportion of Tregs in total CD4+ T cells increased (Fig. 8A), their absolute numbers decreased significantly in the spleens of mice that received HSCs (Fig. 8B). Interestingly, Tregs increased significantly in lymph nodes of mice that received HSCs, the effect being greater when LPS-pretreated HSCs were transferred (Fig. 8A, 8B). Immunostaining for Ki67 showed proliferation of Foxp3+ Tregs in the lymph nodes (Fig. 8C), but not spleens (data not shown). Tregs were found in close proximity of adoptively transferred GFP+ HSCs in the lymph nodes and spleen on day 2 (Fig. 8D, 8E), and a small number could be observed in lymph nodes even on day 5 (data not shown). These data indicate the potential of HSCs to recruit and expand Tregs in vivo.

**Discussion**

CD4+CD25+Foxp3+ Tregs suppress the activation/proliferation of autoreactive CD4+ and CD8+ T cells and control allograft rejection, infection-induced immune responses, and inflammatory diseases (49). Expansion of Tregs in a murine model of spontaneous liver allograft acceptance and their potential role in hepatic immune tolerance (50) have been reported. Thus, the differential effects of perisinusoidal HSCs observed in this study (apoptosis of naive conventional CD4+ T cells and expansion of Tregs) indicate that HSCs may play a critical role in liver allograft acceptance/tolerance, especially because the endothelial barrier is disrupted due to cold-ischemic storage and reperfusion of the graft (24, 25). However, even when the sinusoidal endothelium is intact, HSCs can interact with cells in the sinusoids via their cytoplasmic processes penetrating through the SEC fenestrations (13). In this regard, hepatocytes that are located beneath HSCs have been shown to interact with T cells through fenestrations in LSECs (51). Indeed, we observed close association of CD4+ T cells with HSCs in vivo and in vitro, which may be due to the expression of ICAM-1 and VCAM by HSCs (Fig. 1) (21, 52, 53). HSCs are also a principal source of fibronectin (54) that can recruit lymphocytes in the reperfused liver graft by interacting with α4β1 (55). Moreover, retinoic acid, stored mainly by HSCs, enhances the expression of integrin α4β7 on naive CD4+ T cells, which causes their homing toward nonlymphoid tissues (56). Furthermore, HSCs produce CXCL9 (MIG) and CXCL10 (IP-10) (data not shown) that can recruit CXCR3+CD4+ T cells following cold liver I/R injury (57). Together, these observations suggest that HSCs may constitute a major hepatic cell type in regulating immune responses. Indeed, the role of HSCs in immune regulation is exemplified by their ability to protect cotransplanted islet allografts from rejection (58) and enhance hepatocyte engraftment (59).

An important consideration regarding the role of HSCs in hepatic immune regulation is their strong reactivity to LPS, levels of which are elevated pre- and posttransplantation (29). In this study, both untreated and LPS-pretreated HSCs induced apoptosis of conventional allogeneic CD4+ T cells. This property of HSCs contrasts with that of KCs, SECs, and hepatocytes that induce the proliferation of CD4+CD25+ T cells (60). Although it is unclear why upregulation of Fas expression on naive conventional allogeneic CD4+ T cells occurred without their activation/proliferation, our data are consistent with inhibition of activation/proliferation, together with apoptosis, of naive conventional CD4+ T cells due to FasL–Fas cross-linking in the presence of TCR ligation (36, 61). The B7-H1–PD-1 pathway has also been reported to be important for the inhibition of T cell proliferation and their apoptosis (37). Fully activated HSCs have...

**FIGURE 6.** Production of cytokines by LPS-stimulated HSCs and in HSC/Treg cocultures. (A) The supernatants from untreated or LPS-pretreated (100 ng/ml) HSCs, or from their cocultures with Tregs, were aspirated after 5 d incubation and the accumulated cytokines measured via Lumexin assay (n = 3). (B) Intracellular IL-6, TNF-α, and IL-10 were stained in unstimulated or LPS-prestimulated (100 ng/ml) HSCs cultured alone or with Tregs for 5 d. The experiment was performed twice with essentially similar results. *p < 0.05, **p < 0.01.
been shown to induce apoptosis of DC-activated CD4^+ T cells, in part via a B7-H1/PD-1–dependent mechanism, as a result of enhanced B7-H1 expression on HSCs and that of PD-1 on DC-stimulated allogeneic CD4^+CD25^- cells (17). However, we did not observe such an effect of HSCs on conventional CD4^+ T cells. It appears from our studies that quiescent HSCs do not induce PD-1 expression on naive conventional CD4^+ T cells (Fig. 3C). Together with the low expression of B7-H1 on quiescent HSCs, this may be the reason why the PD-1–B7-H1 pathway did not play a role in quiescent HSC-induced apoptosis of naive conventional CD4^+ T cells. Our results indicate that Fas–FasL interaction may be a key mechanism underlying quiescent HSC-induced elimination of conventional allogeneic CD4^+ T cells. Thus, our results indicate that Fas–FasL interaction may be a key mechanism underlying quiescent HSC-induced apoptosis of naive conventional allogeneic CD4^+ T cells. Our data also support the concept (62) that auto- or alloreactive T cells in target organs are activated mainly by nonprofessional APCs and deleted by apoptosis through the Fas/FasL pathway in the absence of adequate costimulation.

It has been reported previously (19) that culture-activated (7–10 d) mouse HSCs (i.e., cells that are retinoid deficient and fibrogenic), once they have been stimulated with IFN-γ for an additional 72 h, cause expansion of Tregs in an IL-2–dependent manner. By contrast, the activation state of HSCs examined in the current study was very low and likely achieved soon after liver transplantation due to the release of free radicals and inflammatory cytokines (14–16). Furthermore, no IFN-γ could be detected in the supernatants of HSC cultures (control or LPS-stimulated) or HSC/Treg cocultures (data not shown). Thus, our data indicate that HSCs can cause Treg expansion both with or without LPS stimulation and in an IFN-γ–independent manner. Considering the distinct effects of LPS and IFN-γ on cytokine production by HSCs, it is reasonable to speculate that the effects of LPS and IFN-γ in causing Treg expansion and promotion of tolerance may be additive or synergistic.

Wiegard et al. (60) reported that KCs, but not SECs or hepatocytes, caused Treg expansion. Interestingly, however, KC-expanded Tregs were less potent than freshly isolated Tregs in suppressing CD4^+ T cell proliferation. In contrast, we found that Tregs expanded by HSCs were much more potent in suppressing CD4^+ T cell proliferation than freshly isolated Tregs. This can be explained by the retention of a suppressive phenotype and enhanced Foxp3 expression by HSC-expanded Tregs (Fig. 7). Additionally, the suppressive activity of Tregs was reversed when KCs were stimulated with LPS (60), whereas the magnitude of suppressor activity of Tregs expanded by untreated and LPS-stimulated HSCs in this study was similar. This observation is interesting because Foxp3 expression is directly linked to Treg function (63), and Tregs from genetically engineered mice that express low levels of Foxp3 lose suppressive activity and gain suppressor function.

**FIGURE 7.** HSCs favor retention of a regulatory phenotype and suppressive activity of proliferating Tregs. (A) Purified Tregs cocultured with HSCs (unstimulated or LPS-stimulated [100 ng/ml]) were recovered, stained, and analyzed for the expression of the indicated molecules by flow cytometry. Shaded histogram, isotype control; solid lines, freshly isolated Tregs; dotted lines, HSC-expanded Tregs; dashed lines, LPS-pretreated HSC-expanded Tregs. (B) HSC-expanded allo-Tregs potently suppress the proliferation of syngeneic conventional CD4^+ T cells. Purified naive conventional B6 CD4^+ T cells (Ly5.2; prestained with CFSE) were cocultured with γ-irradiated BALB/c splenocytes (at a ratio of 10:1). Freshly isolated B6 Tregs or Tregs recovered from cocultures with untreated or LPS-pretreated BALB/c HSCs were added at the indicated ratios to the conventional CD4^+ T cell/splenocyte coculture. As negative controls, no Tregs or naive or anti-CD3/CD28 Ab-activated conventional B6 CD4^+ T cells (coexpressing Ly5.1 and 5.2) were added at 1:10 ratio. Following incubation for 96 h, proliferation was measured in CD45.2^+ CD4^+ T cells, while excluding Foxp3^- as well as CD45.1 and CD45.2 double-positive cells. The numbers in the panels indicate the incidence of proliferating CD4^+ T cells as a percentage of total CD4^+Foxp3^- cells (n = 2). (C) In vivo stability of the HSC-expanded Tregs following their adoptive transfer (measured on the basis of their Foxp3 expression). Freshly isolated, BALB/c HSC-expanded (untreated or LPS-pretreated), and anti-CD3/CD28-expanded B6 Tregs were labeled with CFSE and adoptively transferred to syngeneic B6 mice (1 × 10^6/animal). At 1 wk, expression of Foxp3 in labeled cells was determined in spleen and lymph nodes using FACS. Numbers in panels denote mean fluorescence intensity. n = 2.
Our study demonstrates an absolute requirement for cell–cell contact and MHC class II dependence for Treg expansion by HSCs. Even though constitutive expression of MHC class II is low on HSCs, it increases significantly upon LPS stimulation, which is consistent with enhanced Treg expansion by LPS-pretreated HSCs. Irla et al. (72) reported recently that MHC class II-dependent, myelin Ag-specific interaction of plasmacytoid DCs with naturally occurring Tregs in lymph nodes caused their expansion during autoimmune encephalomyelitis. The role of Tregs in this latter model was indicated by exacerbated pathology in MHC class II-deficient mice. Mast cells have also been shown to stimulate proliferation of naturally occurring Tregs in an MHC class II-dependent manner (73). Experimental evidence also suggests the role of coregulatory molecules (for instance CD80/CD86 and B7-H1) in Treg expansion (74, 75). However, almost complete inhibition of HSC-induced Treg expansion by anti-MHC class II Ab suggests predominance of TCR ligation in this phenomenon. Moreover, mast cell-induced Treg expansion is also unaffected by anti-B7-H1 Ab (73). Although our data demonstrate that MHC class II-dependent expansion of Tregs by HSCs may be an important mechanism of hepatic immune regulation, we note that KCs also express MHC class II constitutively (73). Experimental evidence also suggests the role of coregulatory molecules (for instance CD80/CD86 and B7-H1) in Treg expansion (74, 75). However, almost complete inhibition of HSC-induced Treg expansion by anti-MHC class II Ab suggests predominance of TCR ligation in this phenomenon. Moreover, mast cell-induced Treg expansion is also unaffected by anti-B7-H1 Ab (73). Although our data demonstrate that MHC class II-dependent expansion of Tregs by HSCs may be an important mechanism of hepatic immune regulation, we note that KCs also express MHC class II constitutively (11). Thus, a similar MHC class II-dependent effect of KCs on Treg expansion cannot be ruled out. However, sinusoidal endothelium is disrupted for a considerable time, and KC as well as DC numbers decrease after reperfusion (76), emphasizing the potential significance of HSCs in liver allograft tolerance, especially during the initial period following graft reperfusion. The homing of Tregs has been shown to be critical for the development of peripheral tolerance and acceptance of heart allografts (77). HSC-expanded Tregs expressed CCR7 and especially CD62L, molecules implicated in homing of Tregs to secondary lymphoid tissues, at a high level (Fig. 7A). These findings are consistent with our in vivo observations in which adoptively transferred HSCs...
enhanced homing/recruitment as well as the expansion of Tregs in the lymph nodes (Fig. 8).

In conclusion, expansion of Tregs and concomitant apoptosis of naive conventional CD4+ T cells by HSCs could favor immunological tolerance after liver transplantation and also be of value for ex vivo expansion of Tregs for potential therapeutic applications.

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Disclosures

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References

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**Supplemental Figure 1: Purity of HSCs.** Freshly-isolated B6 HSCs were purified, and stained for CD68 (Kupffer cell marker), CD11b (Kupffer cell and myloid cell marker), CD11c (dendritic cell and natural killer cell marker) and desmin (HSC marker), and analyzed by flow cytometry. Lower panel shows the isotype controls.