Published in final edited form as:

Transplantation. 2008 December 15; 86(11): 1492–1502. doi:10.1097/TP.0b013e31818bfd13.

Hepatic Stellate Cells Preferentially Expand Allogeneic CD4⁺CD25⁺FoxP3⁺ Regulatory T Cells in an IL-2 Dependent Manner

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Abstract

Background—Organ transplantation has been successfully practice for decades, but the outcome of cell transplantation remains disappointing. This is the case in animal models; liver allografts in mice are spontaneously accepted without requirement of immunosuppression, whereas hepatocyte transplants in the same combination are acutely rejected, apparently resulting from immune attacks because syngeneic hepatocyte transplants survive indefinitely. This suggests that liver non-parenchymal cells play an important role in protecting parenchymal cell from rejection. We have shown that hepatic stellate cells (HpSC), well known to participate in liver repairing and fibrosis, mediate potent immunomodulatory functions via induction of activated T cell death.

Methods and Results—Here we report that HpSC acquired antigen presenting capacity following activation by IFN- γ . In contrast to professional APC dendritic cells (DC) that predominantly stimulated CD4⁺ T cells to generate CD25⁺Foxp3[−] effector cells, HpSC selectively expanded CD4⁺CD25⁺Foxp3⁺ cells in an IL-2 dependent manner. These expanded CD4⁺CD25⁺Foxp3⁺ cells showed regulatory T (Treg) cell activity in effectively inhibiting T cell proliferation in responses to anti-CD3 mAb or alloantigens in a MHC non-specific fashion. The Treg cells were expanded from the CD4⁺CD25⁺ population with the help of IL-2, independent of B7-H1 and TGF- β . Administration of HpSC into allogeneic recipients resulted in expansion of CD4⁺CD25⁺FoxP3⁺ cells in vivo.

Conclusion—Liver stromal HpSC acted as non-professional APC, and preferentially expanded CD25⁺FoxP3⁺ Treg cells, which may contribute to immune regulation in the liver.

Keywords

Hepatic stellate cell; Regulatory T cell; Dendritic cell; Antigen Presentation; IL-2; B7-H1; TGF- β ; Immune regulation; T cell response

Introduction

Organ transplantation has been successfully practice for decades, but the outcome of cell transplantation remains disappointing. This is the case in animal models; liver allografts in many species are spontaneously accepted without requirement of immunosuppression (1–3).

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¹This study was supported by funds from National Institute of Health Grant DK58316 (SQ) and Roche Organ Transplantation Research Foundation (LL).

Portal venous infusion or oral administration of an antigen (4,5) leads to antigen-specific tolerance (4,5). However, hepatocyte transplants are acutely rejected, apparently resulting from immune attacks because syngeneic hepatocyte transplants survive indefinitely (6). This suggests that liver non-parenchymal cells play an important role in protecting parenchymal cell from rejection.

We have recently tested this hypothesis in mouse liver stromal cells, called hepatic stellate cells (HpSC) that are well known to participate in the reparative processes following liver injury. HpSC, upon activation, possess potent immunomodulatory activity. They markedly suppressed allogeneic T cell immune responses via induction of T cell apoptosis, which was partially mediated by B7-H1 expressed on HpSC (7). Co-transplantation of allogeneic islets with HpSC effectively protected islet allografts from rejection (8). In this study, we demonstrate that HpSC, upon activation by interferon (IFN)- γ , a key cytokine released by activated T cells during inflammation, acquire the ability to present antigens and selectively expanded CD25⁺ forkhead box P3 (FoxP3)⁺ T regulatory cells (Treg) in an IL-2 dependent manner.

Materials and Methods

Animals

Male C57BL/6J (B6; H-2^b) and BALB/c (H-2^d) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Thy1.1⁺ (BALB/c background) and B7-H deficient (B6 background) mice were kindly provided by Drs. Hyam I. Levitsky and Lieping Chen of Johns Hopkins University Medical School (Baltimore, MD), respectively. All animals were maintained in the specific pathogen-free facility at the Lerner Research Institute, Cleveland Clinic (Cleveland, OH). All animals received care according to the criteria of NIH.

Preparation of HpSC

HpSC were isolated from mouse liver NPC as previously described (7), and cultured (10⁵/ml) in cell culture flask (25 cm² surface area) (Nunc™, Roskilde, Denmark) with RPMI-1640 (Mediatech Inc., Herndon, VA) supplemented with 20% fetal calf serum (FCS) in a 5% CO₂ in air at 37°C for 7–10 days. Cell viability was greater than 90% as determined by trypan blue exclusion. The purity of HpSC was determined by desmin immunostaining and the typical light microscopic appearance of the lipid droplets. Activation of HpSC was accomplished by incubation with 200 U/ml IFN- γ (R & D Systems, Minneapolis, MN) for the last 72 hours of culture (subsequently referred to as γ -HpSC).

Culture of DC

B6 bone marrow cells were cultured in complete RPMI-1640 medium containing 10% FCS in the presence of recombinant mouse GM-CSF (4 ng/ml) and IL-4 (1000 U/ml) (both from Schering-Plough, Kenilworth, NJ) as previously described (9). DC were isolated from non-adherent cells released from the proliferating cell clusters and purified by positive sorting with CD11c mAb coated magnetic beads (Milteny Biotec, Auburn, CA).

mAb and Flow cytometry

To examine expression of surface molecules, cells were stained with mAbs against CD3, CD4, CD25, CD40, CD54, CD80, CD86, MHC class I (H-2^b), II (I-A^b), Thy1.1 (BD. PharMingen, San Diego, CA) or B7-H1 antigen (eBioscience, San Diego, CA). Staining for FoxP3 was performed using an intra-cellular staining kit (eBioscience). Appropriate isotype control antibodies were used in all experiments. For CFSE dilution assay, T cells (10⁷/ml) were

incubated with 0.5 μ M CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C. Cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

T cell purification

CD4⁺ T cells were purified using magnetic beads via positive or negative (bead-coated cocktail antibodies) selection with >99% purity as determined by flow cytometry. CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were purified using magnetic bead-coated anti-CD25 mAb with purity >99%.

Proliferation and suppression assays

For proliferation assay, responder T cells (2×10^5 /well in 100 μ l) were cultured with graded doses of γ -irradiated stimulator cells [40Gy for HpSC to completely inhibit their proliferation (7), 20Gy for DC and spleen cells] in triplicate in 96-well plates, and maintained in RPMI-1640 complete medium for 3 days in 5% CO₂ in air. [³H]TdR (1 μ Ci/well) was added for the final 18 h, and incorporation of [³H]TdR was assessed by liquid scintillation counting. Results are expressed as mean cpm $\pm$ 1SD. To determine the inhibitory activity, purified CD4⁺CD25⁺ or control cells were added, at indicated ratio, into the reporter T cells (10^5 /well in 100 μ l) which were stimulated by anti-CD3 mAb (2 μ g/ml) in the presence of APC. In some experiments, T cell proliferation was determined by CFSE dilution assay. Multiple cytokine levels in the culture supernatants were determined with cytometric bead array (CBA) kit (BD PharMingen) according to the manufacturer's instructions.

Quantitative Real-time PCR

Total RNA was purified using TRIzol reagent. 1 μ g aliquot of RNA was treated with deoxyribonuclease I (Invitrogen, Carlsbad, CA). The specific primers for Foxp3 (forward: GGC CCT TCT CCA GGA CAG A, reverse: GGC ATG GGC ATC CAC AGT) and 18S rRNA controls were used in quantitative real-time PCR in duplicates using an ABI Prism 7500 machine (Applied Biosystems). The cycle quantity was determined with a standard curve, and expression levels were normalized to 18S rRNA.

Statistical analysis

The parametric data were analyzed by Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

HpSC stimulate proliferative responses in allogeneic T cells

Flow analysis revealed that HpSC constitutively expressed MHC class I, CD54, and were negative for MHC class II and CD86. Following exposure to IFN- γ , a key cytokine produced by activated T cells, MHC class I and CD54 expression were markedly upregulated, and MHC class II and B7-H1 became detectable, although CD40 and CD86 remained low (Fig. 1A). Co-culture of non-activated HpSC barely induced allogeneic T-lymphocyte proliferation, while γ -HpSC elicited significant T cell proliferation, which was validated by [³H]TdR uptake and CFSE dilution assays (Fig. 1B and C). However, γ -HpSC induced less robust division of T cells compared with DC that stimulated vigorous T cell proliferation ($p < 0.05$). As shown in Figure 1D, DC-stimulated CD4⁺ T cells produced abundant IL-2 and IFN- γ , as well as IL-10, whereas substantially lower levels of IL-2 and IFN- γ were detected in cultures with γ -HpSC (all $p < 0.05$), suggesting weak T cell activation or induction of suppressor T cells. A higher expression of FoxP3 mRNA, a critical transcription factor of Treg cells (10–12), was detected by qPCR in γ -HpSC stimulated-CD4⁺ T cells (Fig. 1E, $p < 0.05$ compared with DC, HpSC or control groups), suggesting possible induction of FoxP3⁺ Treg cells.

γ -HpSC preferentially expand CD25⁺FoxP3⁺ cells

To determine the capacity of γ -HpSC to expand CD25⁺FoxP3⁺ cells, purified BALB/c CD4⁺ T cells (2×10^5) were co-cultured with irradiated B6 HpSC, γ -HpSC or DC at a ratio of 10:1 for 5 days, then double stained with anti-CD25 and anti-FoxP3 mAbs. Flow data showed that naive BALB/c CD4⁺ T cells contained 6.5% CD25⁺FoxP3⁺ cells (Fig. 2A), reflecting the presence of naturally occurring Treg cells (13), and this population decreased to 2.1% after 5 days culture of CD4⁺ T cells alone, presumably due to lack of stimulation. However, CD25⁺FoxP3⁺ cells in the culture with γ -HpSC increased to 14.8% (Fig. 2A). CFSE dilution analysis gated on CD25⁺FoxP3⁺ cells showed that these cells were actively dividing (Fig. 2A, upper panels), suggesting that CD25⁺FoxP3⁺ cells are expanded by γ -HpSC stimulation. The proportion of CD25⁺FoxP3⁺ cells in non-activated HpSC group remained 2.4%, similar to that of CD4⁺ T cells culture alone, without proliferation (Fig. 2A, upper panels), indicating IFN γ -activation was required for HpSC to expand CD25⁺FoxP3⁺ cells. Whereas, DC actively stimulated allogeneic CD4⁺ T cell proliferation, leading to expansion of CD25⁺ cells up to 60%, of which however, 89% were FoxP3⁻ (Fig. 2A).

Immunomodulatory effect of CD4⁺CD25⁺ cells elicited by γ -HpSC

It was of importance to determine the function of the γ -HpSC driven CD25⁺FoxP3⁺ cells. However, due to intra-cellular staining of FoxP3 required cell membrane permeation which impaired the function of the CD25⁺FoxP3⁺ cells via sorting purification. An alternative approach was to examine the function of total CD4⁺CD25⁺ population since more than 85% of them were CD25⁺FoxP3⁺ following γ -HpSC stimulation (Fig. 2A), which would be compared to CD4⁺CD25⁻ population. CD25⁺ and CD25⁻ T cells purified from BALB/c CD4⁺ T cells and B6 γ -HpSC co-culture were added into the reporter BALB/c CD4⁺ T cells in the presence of anti-CD3 mAb and irradiated BALB/c spleen cells (providing co-stimulation). As shown in Fig. 2B, CD4⁺CD25⁺ T cells themselves were anergic to anti-CD3 stimulation and were capable of inhibiting proliferation of the reporter T cells. CD4⁺CD25⁻ did not show suppressor effect. The CD4⁺CD25⁺ cells also effectively suppressed proliferative response of CD4⁺CD25⁻ T cells (Fig. 2B). An increase in CD4⁺CD25⁺ cell doses resulted in enhanced inhibitory effect (Fig. 2C). These data suggest that γ -HpSC-expanded CD4⁺CD25⁺ cells contain Treg cells. In contrast, CD25⁺ T cells isolated from DC and CD4⁺ T cells co-culture, most of which were Foxp3⁻ (89%, Fig. 2A), did not inhibit T cell proliferation (Fig. 2D). To verify whether the inhibitory activity of γ -HpSC-expanded Treg was MHC specific, the BALB/c (H-2^d) CD4⁺CD25⁺ cells expanded by B6 (H-2^b) γ -HpSC were added into CFSE-labeled BALB/c T cells whose proliferation was elicited by splenocytes from B6 or C3H (H-2^k, third party strain). As noted in Fig. 2E, B6 γ -HpSC expanded CD4⁺CD25⁺ cells demonstrated comparable inhibitory effect on T cell proliferation induced by either B6 or C3H spleen cells, indicating that the inhibitory effect is not MHC specific.

CD25⁺FoxP3⁺ Treg are expanded from the CD4⁺CD25⁺ population in an IL-2 dependent manner

To determine the role of IL-2 in expansion of CD25⁺FoxP3⁺ cells, IL-2 was neutralized by addition of anti-IL-2 mAb (10 μ g/ml) into γ -HpSC and CD4⁺ T cells culture. CD25⁺FoxP3⁺ expansion was almost total blocked, indicating a crucial role of IL-2 (Fig. 3A). To ascertain whether these expanded CD25⁺FoxP3⁺ Treg originated from CD4⁺CD25⁺ cells or were converted from the CD4⁺CD25⁻ population, B6 γ -HpSC were cultured with CFSE-labeled bulk CD4⁺, CD4⁺CD25⁻ or CD4⁺CD25⁺ cells purified from normal BALB/c spleen. At the end of cultures, IL-2 levels in the supernatant were assessed by CBA. Expansion of Treg was determined by CFSE dilution gated on Foxp3⁺ populations. In the bulk CD4⁺ cell group, FoxP3⁺ cells showed marked proliferation (Fig. 3A) with IL-2 levels of 82.8 ± 2.1 pg/ml. There was no proliferation of FoxP3⁺ cells in either CD4⁺CD25⁺ (IL-2 was undetectable) or

CD4⁺CD25⁻ group (with IL-2 levels of 77.9±11.4 pg/ml). Proliferation of FoxP3⁺ cells in CD4⁺CD25⁺ group was evident in the presence of exogenous IL-2 (100 U/ml). Without γ -HpSC, IL-2 alone could not drive proliferation of CD25⁺ FoxP3⁺ cells. No generation of FoxP3⁺ cells was seen in CD4⁺CD25⁻ group with exogenous IL-2 (Fig. 3B). Taken together, these data suggest that the FoxP3⁺ cells are expanded from CD4⁺CD25⁺ cells, which requires IL-2. They are not converted from CD4⁺CD25⁻ cells. To further confirm this, we used Thy1.1⁺ mice. B6 γ -HpSC were co-cultured with BALB/c CD4⁺ T cells, in which CD25⁻ cells purified from normal BALB/c mice (Thy1.1⁻) were mixed with Thy1.1⁺ CD25⁺ cells at 10:1 ratio. The CD25⁺FoxP3⁺ cells were increased to 13.7% following 5 days culture. 95.2% of the FoxP3⁺ cells were Thy1.1⁺ (Fig. 3C), although, in the culture, vast majority of CD4⁺ T cells were CD25⁻ (Thy1.1⁻), indicating that expanded CD25⁺FoxP3⁺ cells are unlikely converted from CD4⁺CD25⁻ cells.

γ -HpSC-induced expansion of CD25⁺FoxP3⁺ CD4⁺ cells is not mediated by B7-H1 or TGF- β

Our previous data demonstrated that T cell death induced by γ -HpSC was partially mediated by the anti-inflammatory molecule B7-H1, which is highly upregulated on activated HpSC (7). To determine whether B7-H1 was also involved in the expansion of CD25⁺FoxP3⁺ cells, a blocking anti-B7-H1 mAb ranging from 5 to 15 μ g/ml was added into the culture of B6 γ -HpSC and BALB/c CD4⁺ T cells. B7-H1 mAb did not abrogate the expansion of CD25⁺FoxP3⁺ cells by γ -HpSC (Fig. 4). Furthermore, γ -HpSC from B7-H1 knockout mice (B7-H1^{-/-}) effectively expanded CD25⁺FoxP3⁺ cells in allogeneic CD4⁺ T cells, similar to that from wild type mice (Fig. 4), suggesting that B7-H1 was not required for Treg expansion by γ -HpSC. To test the role of TGF- β 1, a serial concentrations of TGF- β 1 neutralizing antibody (0.1, 0.5, 1, 5, 10 or 15 μ g/ml) was added into the culture of B6 γ -HpSC and BALB/c CD4⁺ T cells. There was no attenuated expansion of CD25⁺FoxP3⁺ cells compared to the controls (addition of mouse IgG) (Fig. 4A). To rule out the possibility that anti-TGF- β 1 mAb might not be sensitive enough to block latent TGF- β , we used SB-525334 (6-[2-ter-butyl-5-(6-methylpyridin-2-yl)-1H-imidazol-4-yl]-quinoxaline, Sigma-Aldrich), a potent and selective inhibitor of the TGF- β 1 receptor, actin receptor-like kinase (ALK5) to inhibit TGF- β 1 receptor signaling. Addition of SB-525334 at 10 μ M (a concentration almost completely inhibited TGF- β -induced phosphorylated Smed-2 expression in NIH3T3 cells) showed no effect on HpSC-induced Treg cell expansion (Fig. 4B), indicating that TGF- β is not a critical factor for CD25⁺FoxP3⁺ cell expansion in this experimental model.

Evidence of expansion of FoxP3⁺ Treg elicited by γ -HpSC in vivo

To gain evidence *in vivo*, γ -HpSC (10^6) from B6 mice were injected intravenously into BALB/c mice. Injections with HpSC, DC or PBS were used for comparison. T cells isolated from spleen or lymph nodes (LN) 5 days later were examined for Treg cells by detection of FoxP3 mRNA with qPCR and by identification of CD4⁺CD25⁺Foxp3⁺ cells with flow cytometry. The levels of FoxP3 mRNA in the LN of γ -HpSC injection group were significantly higher compared to those from HpSC or DC injection groups (Fig. 5A, $p < 0.05$, γ -HpSC vs. HpSC or DC groups), suggesting that γ -HpSC might elicit FoxP3⁺ cell expansion. Lymphocytes from the spleen were triple stained with mAbs against CD4, CD25 and FoxP3 for FACS analysis. The percentage of CD25⁺FoxP3⁺ cells in CD4⁺T cell population increased from 5.9% (in PBS control) or 6.3% (in HpSC group) to 13.4% (in γ -HpSC group), indicating that IFN- γ activation markedly enhanced the ability of HpSC to expand Treg *in vivo* (Fig. 5B). T cell responses of those immunized mice to alloantigens were assessed *ex vivo*. When LN T cells of immunized mice were restimulated *in vitro* with irradiated B6 spleen cells, a robust proliferative response of T cells was observed in the DC group, while T cell proliferation in γ -HpSC group was significantly lower (Fig. 5C, $p < 0.05$, γ -HpSC vs. HpSC or DC group). These results suggest that *in vivo* administration of γ -HpSC leads to expansion of Treg and induction of T cell hyporesponsiveness.

Discussion

Many studies have suggested that the liver adapts its immune response to a balance to avoid uncontrolled activation. Although the underlying mechanisms are not completely understood, the importance of antigen presentation by constitutive parenchymal and non-parenchymal cells in the liver in regulating immune responses is being increasingly recognized (14,15). In this study, we demonstrate that HpSC, as nonprofessional APC, have a limited role in initiating T cell activation: they do not constitutively express the molecules required for antigen presentation, including MHC class II, and costimulatory molecules. However, these molecules can be induced upon inflammation and in response to various cytokines. Upon activation by inflammatory cytokines, including IFN- γ , HpSC become MHC class II positive, and capable of stimulating allogeneic CD4⁺ T cells. This is consistent with previous reports that non-professional APC usually acquire their function upon stimulation with pro-inflammatory cytokines (15,16,17–18). Several recent studies have also shown that HpSC are capable of presenting endogenous and exogenous antigens (16,19), similar to a number of other cell types in the liver, including sinusoidal endothelial cells that acquire antigen presenting function upon stimulation with cytokines (20–22). Indeed, cells in other tissues, such as brain astrocytes or kidney mesangial cells, also displayed features of nonprofessional APC under certain conditions (23,24).

The data of this study also demonstrate that the IFN- γ activated HpSC preferentially expand CD25⁺Foxp3⁺ Treg cells with strong immune regulatory activities both *in vitro* and *in vivo*, suggesting a possible role of HpSC in immune regulation in the inflammatory liver. Currently, we are not able to directly test whether HpSC play an important role in induction of T cell hyporesponsiveness in the spontaneously accepted mouse liver allografts which has recently been shown to be associated with markedly increased CD4⁺CD25⁺Foxp3⁺ Treg cells in both liver grafts and recipient spleen (25), due to lack of approaches to specifically inhibit or delete HpSC in the liver. Consistent with many other reports that Treg cells inhibit activation of T cells in an antigen non-specific manner (26,27), our data also show that HpSC expanded Treg cells that suppressed T cells responses to allo-antigens in a non-MHC specific manner. Interestingly, in contrast to HpSC, professional APC predominantly expand CD4⁺CD25⁺Foxp3[−] T cells. Thus, allogeneic T cells primed by DC produce Th1 cytokines IL-2 and IFN- γ , as well as IL-10. IL-10 was originally recognized as a Th2 cytokine, recent papers demonstrated that IL-10 is produced by CD4⁺CD25[−]FoxP3[−] Th1, rather than Th2 cells (28–30). Although it remains unclear what cause the differences between DC and HpSC in directing T cell differentiation, the distinct surface key molecule expression and cytokine profile may contribute to their different activity. Activated HpSC, unlike DC, express low levels of CD40, CD86 (Fig. 1), and do not produce IL-12 (7,27), which may affect their antigen presentation pattern resulting in eliciting Treg cell differentiation.

It is controversial whether Treg cells are expanded from CD4⁺CD25⁺ or converted from CD4⁺CD25[−] cells (31,32). In our experimental model, the CD25⁺FoxP3⁺ cells expanded by γ -HpSC appeared to be directly derived from CD4⁺CD25⁺ T cells (Fig. 3A and B). The current study also demonstrated that the expansion of CD25⁺FoxP3⁺ cells was dependent on IL-2. IL-2 was detected in the supernatant of CD4⁺ cells/ γ -HpSC culture, neutralizing IL-2 totally blocked Treg expansion. In the CD4⁺CD25⁺ cells/ γ -HpSC culture, there was no Treg expansion and no IL-2 detection. However, Treg expansion is evidenced, when exogenous IL-2 was added into the culture (Fig. 3A). These data indicate that γ -HpSC induced expansion of Treg is IL-2 dependent, and CD4⁺CD25[−] cells may be the source of IL-2 in γ -HpSC and CD4⁺ T cell culture. A recent study has showed that retinoid acid enhances Treg cell growth and differentiation (33). HpSC are main retinol storage cells, and produce huge amount of retinoid acid (34,35). However, our data reveal that blockage of IL-2 alone almost completely inhibits expansion of Treg cells, therefore retinoid acid is unlikely to play a crucial role in

induction of Treg cells in this experimental system. Two key ingredients of Treg conversion from CD4⁺CD25⁻ T cells in mice are TGF- β and either IL-2 or retinoic acid (33). Here we show by neutralization of TGF- β with anti-TGF β mAb or blockade of TGF- β signaling with ALK5 inhibition (Fig. 4A and B) that TGF- β is not involved in HpSC-induced Treg cell expansion. This may explain why HpSC can only expand pre-existing Foxp3⁺ Treg cells, not convert Treg from CD4⁺CD25⁻ T cells. In addition, it has also been reported that promoting CD4⁺CD25⁻ T cell differentiation into CD4⁺CD25⁺ Treg cells is dependent on CD86 expression on APC (36). Some costimulatory molecules (CD86 and CD40) are low expressed in activated HpSC. γ -HpSC appear to lack the elements, which are required for CD25⁺FoxP3⁺ Treg conversion from CD4⁺CD25⁻ T cells.

Our findings support a model, in which an active immune response is initially provoked by acute antigen presentation in the liver, as might occur with hepatic infection or liver allograft transplantation. In the acute phase of immune response, IFN- γ and IL-2 released by activated T cells may serve as key factors in supporting expansion of Treg by activation of HpSC in the liver. In cases of antigen persistence, the immune responses are modulated by regulatory systems over time, ultimately resulting in tolerance. This is reflected by the strong initial immune responses of alloreactive lymphocytes in the liver followed by liver allograft acceptance or donor antigen tolerance in several animal models (37).

These data, taken with our previous reports, suggest a crucial role of tissue cells, such as HpSC in the liver in modulation of immune responses in inflammation environments. In response to inflammatory cytokines HpSC are capable of inhibiting naive T cell activation by induction of Treg activity in the presence of IL-2. In addition, HpSC induce apoptosis of activated T cells which is partially mediated by B7-H1 (7,8). These mechanisms may contribute to establishment of hepatic tolerance, which was initially demonstrated by spontaneous acceptance of liver allografts in many species (1–3).

Acknowledgments

We thank Dr. Liping Chen for providing B7-H1 knockout mice, Kathleen Brown for assistance for assay procedures.

Abbreviations

DC	dendritic cell
APC	antigen presenting cells
FoxP3	forkhead box P3
IFN- γ	gamma interferon
HpSC	hepatic stellate cells
γ -HpSC	gamma interferon stimulated hepatic stellate cells
MLR	mixed lymphocyte reaction
KO	knockout
NPC	nonparenchymal cells
Treg	T regulatory cells
Th1	T helper 1
LN	lymph node
[³ H]TdR	tritiated thymidine

mAb	monoclonal antibody
PBS	phosphate buffered saline
qPCR	quantitative polymerase chain reaction
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid

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(A)

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(D)

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Figure 1. Hepatic stellate cells are capable of presenting antigens to allogeneic T cells

(A) Key molecule expression on hepatic stellate cells (SC), IFN- γ -stimulated hepatic stellate cells (γ -SC) and bone marrow-derived dendritic cells (DC) from B6 (H2^b) mice. Expression of MHC class I, II, CD40, CD80, CD86, B7-H1 and CD54 was displayed as filled histograms analyzed by flow cytometry (empty histograms were isotype controls). (B and C) γ -SC are capable of stimulating proliferative response in allogeneic T cells. (B) In a one way MLR assay, BALB/c (H2^d) spleen T cells (2×10^5) were cultured with graded doses (as indicated) of irradiated SC, γ -SC (both 40 Gy) or DC (20 Gy) from B6 mice for 3 days. T cell proliferation was determined by thymidine uptake. (C) CFSE labeled CD4⁺ T cells (2×10^5) purified from BALB/c spleen cells were cultured with irradiated SC, γ -SC or DC from B6 mice at a ratio of 10:1 for 3, 5 or 7 days. Proliferative response of CD4⁺ T cells was analyzed by CFSE dilution assay. (D) Cytokine profile. The supernatant cytokine levels were assessed following 5 days cultures of BALB/c CD4⁺ T cells with B6 SC or γ -SC (lower panel) or DC (upper panel) using CBA kits. (E) Fc γ R3 mRNA expression. RNA was isolated from BALB/c CD4⁺ T cells harvested at the end of 5 day-cultures with B6 SC, γ -SC or DC, and examined for Fc γ R3 mRNA expression by quantitative PCR. The data are representative of three separated experiments.

(C)

(D)

(E)

Figure 2. Hepatic stellate cells selectively expand CD25⁺FoxP3⁺ Treg cells

(A) Selective expansion of CD25⁺Foxp3⁺ cells by IFN- γ -stimulated hepatic stellate cells (γ -SC). CFSE labels CD4⁺ T cells (2×10^5) purified from BALB/c (H2^d) spleen cells were cultured with irradiated (40 Gy) B6 (H2^b) SC, γ -SC at a ratio of 10:1 for 5 days. Fresh BALB/c CD4⁺ T cells, cultured alone or stimulated by irradiated (20 Gy) B6 dendritic cells (DC) at 10:1 ratio for 5 days, served as controls. At the end of the culture, cells were double stained with mAbs to CD25 and Foxp3 molecules for FACS analysis. Cell expansion was determined by CFSE dilution assay in gated CD25⁺Foxp3⁺ cells (Upper panel). (B) Expanded CD4⁺CD25⁺ cells inhibit T cell proliferative responses. CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells purified from a culture (5 days) of BALB/c CD4⁺ T cells with B6 γ -SC were used as the suppressors, and added into a culture of BALB/c CD4⁺ or CD4⁺CD25⁻ T cells (1×10^5) at 1:1 ratio in the presence or absence of anti-CD3 mAb (2 μ g/ml) and irradiated BALB/c (syngeneic) spleen cells. The inhibitory activity of suppressor cells was determined by [³H]TdR uptake by reporter cells. The CD4⁺ T cells alone without anti-CD3 mAb and CD4⁺CD25⁺ T cells alone with anti-CD3 were used as controls. (C) Increase in suppressor cell dose resulted in enhanced inhibitory effect. The number of γ -SC expanded CD4⁺CD25⁺ cells was adjusted to a suppressor: responder ratio of 2:1, the proliferation of responder cells was significantly enhanced ($p < 0.05$). (D) CD4⁺CD25⁺ T cells expanded by DC were not suppressive. CD4⁺CD25⁺ T cells purified from a 5-day culture of BALB/c CD4⁺ T cells with B6 DC (DC/T 1:10) were added at 1:1 ratio into BALB/c CD4⁺ T cells in the presence of anti-CD3 mAb (2 μ g/ml) and irradiated BALB/c spleen cells. The proliferative response was determined by [³H]TdR uptake. (E) The inhibitory activity of CD4⁺CD25⁺ cells is not MHC-specific. The CD4⁺CD25⁺ or CD4⁺CD25⁻ cells purified from a culture of BALB/c CD4⁺ T cells and γ -irradiated B6 γ -SC at a ratio of 10:1 for 5 days were added into a one way MLR culture in which proliferation of the CFSE labeled responder BALB/c T cells (1×10^5) was elicited by irradiated B6 or C3H (H2^k, third party) spleen cells (the ratio of suppressor:stimulator:responder = 1:1:1). T cell proliferation was determined by CFSE dilution assay. The data are representative of two separated experiments.

(B)

(C)

Figure 3. CD25⁺Foxp3⁺ cells are expanded from CD4⁺CD25⁺ population, which requires IL-2
 (A) Neutralization of IL-2 eliminates expansion of CD25⁺FoxP3⁺ cells elicited by IFN- γ -stimulated hepatic stellate cells (γ -SC). CFSE labeled BALB/c (H-2^d) CD4⁺ T cells (2×10^5) were cultured with irradiated (40 Gy) B6 (H-2^b) γ -SC at a ratio of 10:1 for 5 days in the presence of anti-IL-2 (10 μ g/ml). Rat IgG_{2a} was used as isotype control. Cells were double

stained with anti-CD25 and -Foxp3, and analyzed by flow cytometry. Cell proliferation was assessed by CFSE dilution in CD25⁺Foxp3⁺ populations. (B) γ -SC expand CD25⁺FoxP3⁺ from CD4⁺CD25⁺ population, which requires IL-2. B6 γ -SC were cultured with 2×10^5 CFSE labeled CD4⁺, CD4⁺CD25⁺ or CD4⁺CD25⁻ cells from BALB/c mice at a ratio of 1:10 for 5 days. Cells were then stained with anti-Foxp3. Proliferation was determined by CFSE dilution in Foxp3⁺ cell populations. To test the effect of IL-2 on Foxp3⁺ cell proliferation, exogenous IL-2 (100 U/ml) was added at the beginning of the cultures in CD4⁺CD25⁺ or CD4⁺CD25⁻ groups. (C) Expansion of CD25⁺Foxp3⁺ cells from CD4⁺CD25⁺ population is demonstrated by using congenic Thy1.1⁺ mice. Flow analysis showed the purity of CD4⁺CD25⁺ cells purified from BALB/c Thy1.1⁺ mice, and CD4⁺CD25⁻ cells purified from normal BALB/c (Thy1.1⁻) mice. Thy1.1⁺CD4⁺CD25⁺ cells were mixed with Thy1.1⁻CD4⁺CD25⁻ cells at a ratio of 1:10 (total 2×10^5 , histogram showed containing of 9.7% Thy1.1⁺ cells), and cultured with irradiated B6 γ -SC at 10:1 ratio for 5 days. Cells were further double stained with anti-Thy1.1 and -Foxp3 for flow analyses, showing that CD25⁺FoxP3⁺ cells was increased to 13.7%, among which 95.2% were Thy1.1⁺. The data are representative of three separated experiments.

(B)

(C)

Figure 4. Hepatic stellate cells-induce expansion of CD25⁺Foxp3⁺ CD4⁺ cells is not mediated by B7-H1 or TGF β

CD4⁺ T cells (2×10^5) purified from BALB/c (H2^d) spleen cells were cultured, at a ratio of 10:1 for 5 days, with irradiated (40 Gy) B6 (H2^b) IFN- γ -stimulated hepatic stellate cells (γ -SC). At the end of the culture, cells were double stained with mAbs against CD25 and Foxp3 for FACS analysis. (A) In the presence or absence of blocking antibody against B7-H1 (15 μ g/ml) or TGF β 1 (10 μ g/ml). RatIgG_{2a} and mouse IgG were used as isotype controls, respectively. γ -SC from B7-H1 knockout mice (B6 background) instead of that from wild type mice were also tested. (B) Inhibition of TGF β receptor signaling does not affect HSC-induced Treg cell expansion. SB525334 alone (10 μ M) and/or anti-TGF β 1 mAb (10 μ g/ml) were added

at the beginning of culture. A representative Western blot depicting that the presence of SB525334 (10 μ M, Sigma-Aldrich) completely inhibited expression of phosphorylated Smed2 in NIH3T3 cells (ATCC) induced by TGF- β (2 ng/ml). The data are representative of two separated experiments.

(B)

Figure 5. Expansion of CD4⁺CD25⁺FoxP3⁺ cells *in vivo* by hepatic stellate cells

BALB/c (H2^d) mice were intravenously injected with B6 (H2^b) IFN- γ -stimulated hepatic stellate cells (γ -SC) (10^6). The mice treated with SC, DC or PBS were used as controls. T cells were isolated from spleen or lymph nodes 5 days later, and were examined for Treg expansion by qPCR and FACS analysis. (A) Enhanced Foxp3 mRNA in lymph nodes, determined by qPCR. The data are expressed as mean $\pm$ 1SD of three experiments. (B) Increased CD25⁺FoxP3⁺ cells in the spleen CD4⁺ T cells. T cells purified from spleen cells were multiple-stained with anti-CD4, -CD25 and -FoxP3 mAbs. The incidences of CD25⁺FoxP3⁺ cells in CD4⁺ T cell populations were determined by FACS analysis. (C) Administration of γ -SC results in hyporesponsiveness of T cells to donor Ag. The isolated LN T cells (2×10^5 /well) from SC, γ -SC, DC or PBS (none) treated BALB/c mice were restimulated with irradiated (20 Gy) B6 spleen cells at a ratio of 1:1 in triplicate plates for 3 days. The proliferative response of the responder cells was determined by thymidine uptake. The data are representative of two separated experiments.