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Hepatic Stellate Cells Preferentially Induce Foxp3$^+$ Regulatory T Cells by Production of Retinoic Acid

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The liver has long been described as immunosuppressive, although the mechanisms underlying this phenomenon are incompletely understood. Hepatic stellate cells (HSCs), a population of liver nonparenchymal cells, are potent producers of the regulatory T cell (Treg)–polarizing molecules TGF-β1 and all-*trans* retinoic acid, particularly during states of inflammation. HSCs are activated during hepatitis C virus infection and may therefore play a role in the enrichment of Tregs during infection. We hypothesized that Ag presentation in the context of HSC activation will induce naive T cells to differentiate into Foxp3$^+$ Tregs. To test this hypothesis, we investigated the molecular interactions between murine HSCs, dendritic cells, and naive CD4$^+$ T cells. We found that HSCs alone do not present Ag to naive CD4$^+$ T cells, but in the presence of dendritic cells and TGF-β1, preferentially induce functional Tregs. This Treg induction was associated with retinoid metabolism by HSCs and was dependent on all-*trans* retinoic acid. Thus, we conclude that HSCs preferentially generate Foxp3$^+$ Tregs and, therefore, may play a role in the tolerogenic nature of the liver. ***The Journal of Immunology*****, 2013, 190: 2009–2016.**

The consequences of the immunosuppressive nature of the liver likely extend to phenomena as diverse as oral tolerance, liver transplant tolerance, and potentially the dysfunction of the T cell compartment in chronic hepatotropic infections, such as hepatitis B and C viruses (HBV and HCV, respectively). However, the mechanisms underlying liver-associated immunosuppression are not well understood. The role of the liver in oral tolerance is highlighted by two key observations: 1) oral tolerance is dependent on blood flow from the intestine to the liver (1), and 2) can be mimicked by direct Ag delivery via the portal vein (2–5). Importantly, oral tolerance may be specifically mediated by the regulatory T cell (Treg) subset (6, 7), indicating a role for tolerogenic interactions between liver APCs and T cells. The tolerogenic nature of the liver also impacts liver transplantation, which can be performed without extensive histocompatibility matching or harsh immunosuppressive regimens, indicating a role for T cell–based tolerance in concert with other well-described, nonadaptive regulatory mechanisms such as immunosuppressive cytokines (8). Liver-associated T cell tolerance may also play a major role in the inability to clear HBV and HCV infections, which are associated with expanded Treg populations in the blood and liver (9–15).

We therefore postulated that Ag presentation in the liver might play a role in the suppression of intrahepatic T cell responses through the induction of Tregs. Recent studies have highlighted the differential ability of various APCs to induce differentiation of the distinct lineages of CD4$^+$ T cells (reviewed in Refs. 16–18). Upon interaction with APCs displaying the cognate Ag, naive CD4$^+$ T cells have the potential to differentiate into multiple distinct lineages: 1) IFN-γ–producing TH1 primed in the presence of IL-12; 2) IL-4–, IL-5–, and IL-10–producing TH2 cells primed in the presence of IL-4 or IL-5; 3) IL-17–producing TH17 cells primed in the presence of TGF-β1 and IL-6; and 4) Tregs capable of inhibiting proliferation and cytokine production in other T cells are primed in the presence of TGF-β1 and all-*trans* retinoic acid (ATRA) (16–18). In particular, some gut-derived APCs specifically prime naive CD4$^+$ T cells to become Foxp3$^+$ Tregs in a TGF-β1– and ATRA-dependent manner (7, 19–21).

Ag presentation to CD4$^+$ T cells in the liver can be mediated by multiple cell types, including professional APCs such as liver-resident dendritic cells (DCs) and Kupffer cells (KCs), the liver-resident macrophages, and is reported to extend to other cells including liver sinusoidal endothelial cells (LSECs) (22) and hepatic stellate cells (HSCs) (23). Liver DCs, KCs, and LSECs have been extensively studied for their Ag-presentation capacity and priming of CD4$^+$ T cells. DCs resident in the liver are immature and produce IL-10 and prime TH2 responses (24), but when activated produce IL-12, priming a TH1 response (25). Similarly, KCs alternatively produce IL-12 or IL-10 in response to different stimuli and, therefore, can prime TH1 or TH2 responses (26, 27). LSECs can induce CD4$^+$ T cell division but inhibit TH1 differentiation (28) and have long been associated with the tolerogenic nature of the liver (29). This effect was recently demonstrated to

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Abbreviations used in this article: ATRA, all-*trans* retinoic acid; DC, dendritic cell; GFAP, glial fibrillary acidic protein; HBV, hepatitis B virus; HCV, hepatitis C virus; HSC, hepatic stellate cell; KC, Kupffer cell; RARα, retinoic acid receptor α; α-SMA, α-smooth muscle actin; Treg, regulatory T cell; UVAF, UV autofluorescence.

be mediated by coinhibitory signals delivered by the interaction of PD-1 on the T cell with PD-L1 on the LSEC (30). HSCs were found to express low levels of MHC class II and the costimulatory molecules CD80 and CD86, but have only recently been described to stimulate $CD4^+$ T cells in an Ag-specific manner (23). Similarly, a recent report demonstrated that IFN-γ–pretreated HSCs expanded Tregs during an MLR (31). However, in this system, HSCs expanded only pre-existing Tregs; they did not generate de novo Tregs from naive $CD4^+$ T cells. To date, little is known of the role for HSCs on $CD4^+$ T cell differentiation during priming. Whether HSCs prime naive $CD4^+$ T cells directly or simply provide context during priming and differentiation is not known.

Interestingly, HSCs have been associated with the production of both ATRA and TGF-β, each a requirement for Treg differentiation. HSCs are the primary storage cell for retinol, or vitamin A (32), which is metabolized to ATRA during activation (33), and activated HSCs have also been shown to produce TGF-β1 (32). Recent reports have demonstrated that HSCs do indeed influence Treg differentiation, but only in trans with strong DC stimulation, adding confusion to the field (34). We therefore tested whether HSCs can influence naive $CD4^+$ T cell differentiation into Tregs, either directly or indirectly, using a murine system.

We found that highly purified HSCs were unable to induce proliferation and cytokine production in naive OT-II transgenic T cells directly. In coculture with DCs and low concentrations of TGF-β1, however, HSCs preferentially induced the expression of Foxp3 in $CD4^+$ T cells. Importantly, this Treg development was dependent on HSC production of ATRA, as antagonism of retinoic acid receptor α (RARα) signaling inhibited the upregulation of Foxp3 on naive OT-II T cells. Thus, we conclude that HSCs preferentially induce Treg differentiation during priming in an ATRA-dependent manner. This finding may provide a mechanism for the tolerogenic nature of the liver and the expansion of Tregs during HBV and HCV infections.

Materials and Methods

Animals

C57BL/6 and BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) and OT-II mice, with a TCR transgene specific for OVA peptides 323–339, on the C57BL/6 background were maintained in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and Institutional Animal Care and Use Committee guidelines.

Liver cell isolation

HSCs were isolated from liver of C57BL/6 mice by perfusion with HBSS, then 0.4% protease (Sigma-Aldrich, St. Louis, MO), followed by 0.01% collagenase (Sigma) solutions in DMEM/F12 medium. After gentle mechanical disruption, the resulting suspension was incubated at 37°C in a 0.4% collagenase solution in DMEM/F12 with 0.5 mg/ml DNAse for 20 min. The digested suspension was filtered through 70-μm mesh and centrifuged 50 × *g* for 3 min. The supernatant was then centrifuged and resuspended in 20% Optiprep (Sigma-Aldrich) diluted in HBSS and overlaid with 11.2% Optiprep, then overlaid with HBSS and centrifuged 1400 × g for 17 min. HSCs were harvested from the interface of 11.2% and HBSS layers, and were washed twice with complete RPMI 1640 with penicillin, streptomycin, and amphotericin B (Lonza, Basel, Switzerland). Splenic DCs were isolated using the CD11c microbeads kit for mouse (Miltenyi Biotec, Bergish Gladbach, Germany).

UV autofluorescence–based sorting of HSCs

After density gradient enrichment, liver nonparenchymal cells were stained with Abs to CD146 (FITC, ME-9F1; Miltenyi Biotec), CD45 (PerCP-Cy5.5, 104; BD Bioscience, San Jose, CA), F4/80 (allophycocyanin, BM8; eBioscience), CD11c (allophycocyanin, N418; eBioscience), and MHC class II (I-A/I-E; Alexa Fluor 700, M5/114.15.2; BD Biosciences), and sorted on a FACSAria Cell Sorter II (BD Bioscience). Cell populations sorted were identified as follows: HSC (UV autofluorescence–positive [$UVAF^+$], $CD45^-$ $CD146^-$), LSEC ($UVAF^-$ $CD45^-$ and $CD146^+$), KC ($UVAF^-$ $F4/80^+$), and DC ($UVAF^-$ $CD11c^+$). For the detection of HSC-associated markers, purified liver cells were stained intracellularly with Abs to glial fibrillary acidic protein (GFAP, FITC, 1B4; BD Bioscience) and α-smooth muscle actin (α-SMA, PE, 1A4; R&D Systems). For α-SMA detection, purified HSCs were cultured for 7 d in complete DMEM and stained with α-SMA PE.

Coculture assay

Naive $CD4^+$ T cells from the spleen and lymph nodes of OT-II mice were enriched using the $CD4^+CD62L^+$ T Cell Isolation Kit II for Mouse (Miltenyi Biotec). A total of 1×10^5 CFSE-labeled, naive OT-II T cells were cocultured for 90 h in $RPMI^+$ 10% FBS with 1×10^5 of the HSCs, $CD11c^+$ DCs, or both (1:1 ratio), in the presence of OVA peptide 323–339 (10 μg/ml) and recombinant mouse TGF-β1 at various concentrations. In some conditions, ATRA (100 nM; Sigma-Aldrich) and/or LE540 (1 μM; Wako Pure Chemical Industries, Osaka, Japan) were used as indicated for the duration of the culture. When using UV-sorted cells, HSC inputs were limited to 2×10^4 cell/well. After 90 h, the OT-II T cells were stained for Foxp3 expression using the PE anti-mouse/rat Foxp3 staining set (eBioscience, San Diego, CA). For transwell experiments, naive OT-II T cells, splenic DCs, and HSCs (either HSCs or DCs were separated by transwell insert as indicated) were cocultured for 90 h in the presence of OVA and TGF-β1.

Intracellular cytokine staining

In vitro cultured OT-II cells were restimulated in an anti-CD3 (clone 145-2C11)/anti-CD28 (clone 37.51; BD Biosciences, San Jose, CA)–coated plate for 4 h in the presence of brefeldin A and monensin, and stained with Abs to Foxp3 (PE, FJK-16s; eBioscience), IL-17 (allophycocyanin, eBio17B7; eBioscience), CD4 (PECy7, GK1.5; eBioscience), and IFN-γ (PE, XMG1.2; BD Biosciences). Stained cells were acquired using an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star, Ashland, OR).

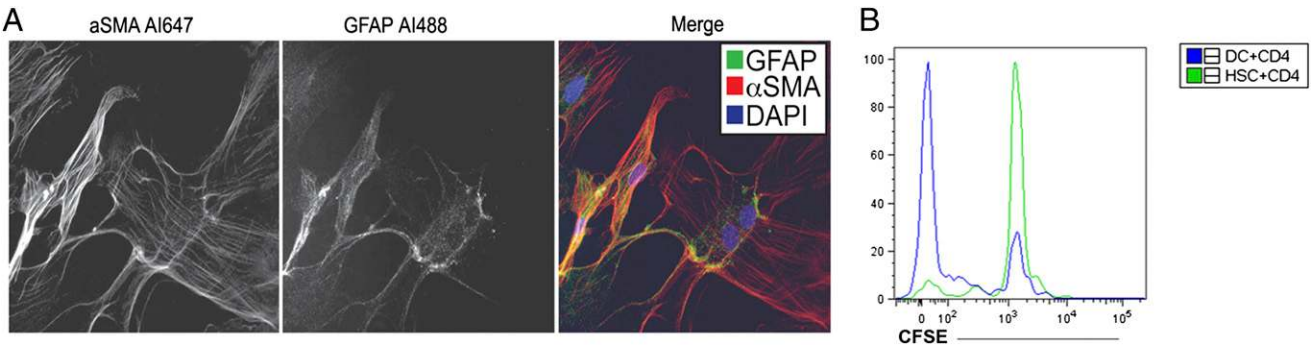

FIGURE 1. HSCs do not stimulate proliferation of naive $CD4^+$ T cells. (**A**) Expression of α-SMA during activation on nontissue culture–treated plates as measured by immunofluorescence microscopy. Images are representative of three independent experiments. (**B**) HSCs from the liver and DCs from the spleen of a C57BL/6 mouse were cocultured individually at a 1:1 ratio (1×10^5 cells each) with purified and CFSE-labeled $CD4^+CD62L^+$ T cells from an OT-II transgenic mouse for 3 d in the presence of the cognate Ag, OVA peptides 323–339 at 10 μg/ml. Representative flow cytometry plots shown were confirmed in more than three independent experiments. Histogram plots shown are gated on live $CFSE^+$ lymphocytes expressing CD4.

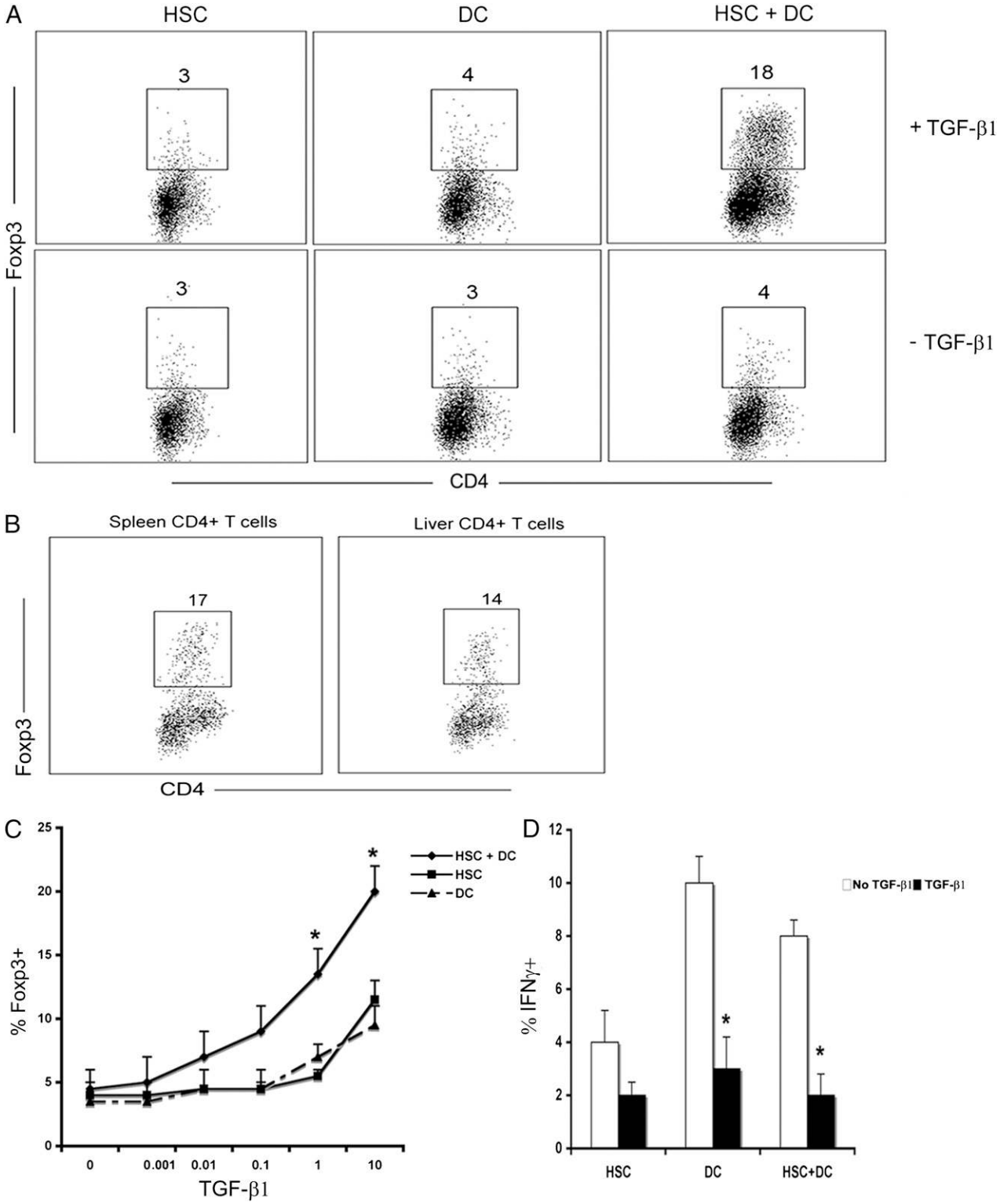

FIGURE 2. HSCs induce Foxp3 expression in the presence of DCs. (**A**) Magnetic bead-isolated OT-II $CD4^+CD62L^+$ T cells were cocultured with the indicated HSCs, DCs, or both for 90 h with OVA peptide 323–339 (10 μg/ml) with or without TGF-β1 (1 ng/ml). Representative flow cytometry plots of Foxp3 expression in directly stained cells. HSCs and DCs were isolated from C57BL/6 mice liver and spleen, respectively. Data are representative of more than three independent experiments. (**B**) Magnetic bead–isolated OT-II $CD4^+CD62L^+$ T cells from spleen and liver as indicated were cocultured with HSCs in the presence of DCs for 90 h with OVA peptide and TGF-β1. Representative flow cytometry plots of Foxp3 expression in directly stained cells. Data are representative of more than two independent experiments. Average percentage of $Foxp3^+$ cells when TGF-β1 is titrated into the culture system (**C**), and percentage of IFN-γ^+ cells (**D**). Titration graphs represent the average of three independent experiments. $*p < 0.05$.

Microscopy

HSCs were plate activated for 7 d, then trypsinized and replated on chambered glass slides for adherence overnight. The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and stained with mouse anti-GFAP mixture (BD Biosciences) and rabbit α-SMA (E184; Novus Biologicals, Littleton, CO), followed by goat anti-mouse Ig (Alexa Fluor 488; Invitrogen, Burlingame, CA) and goat anti-rabbit Ig (Alexa Fluor 647; Invitrogen). Images were acquired using a Zeiss Axioscope Z.1. For autofluorescence microscopy, isolated cells were plated on nontissue culture–treated plates, and images were collected in phase-contrast bright field and under UV excitation.

RT-PCR

RNA was prepared from the indicated cell populations using the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA synthesized using the First-Strand cDNA Synthesis kit using SuperScript II RT with random primers (Invitrogen). cDNA was used as a template for quantitative real-time PCR using SYBR Green Master Mix (BioRad) and the following primers: (all 5′→3′) *Gapdh* sense: TGGCAAAGTGGAGATTGTTGCC; *Gapdh* anti-sense: AAGATGGTGATGGGCTTCCCG; *Aldh1a1* sense: ATGGTTTA-GCAGCAGGACTCTTC; *Aldh1a1* anti-sense: CCAGACATCTTGAATC-CACCGAA; *Aldh2a1* sense: GACTTGTAGCAGCTGTCTTCACT; *Aldh2a1* anti-sense: TCACCCATTTCTCTCCCATTTCC. PCR and analysis were performed using a MyiQ iCycler (BioRad). Gene expression was calculated relative to *Gapdh*.

Results

HSCs do not stimulate proliferation of naive CD4$^+$ T cells

HSC, also known as lipocytes for their prominent lipid vesicles, are buoyant in comparison with other liver cells and can thus be enriched by density gradient centrifugation, the first step in the standard technique for HSC isolation (reviewed in Ref. 32). We performed isolation of HSCs by this method and tested the enrichment of HSCs by analyzing morphology and signature molecule expression following culture on nontissue culture–treated plastic to induce activation. The isolated cells adhered rapidly, lost lipid content, and underwent differentiation into fibroblast-like cells (data not shown), consistent with reports of HSC activation in vitro (32). Subsequent immunofluorescence staining for GFAP, a molecule expressed in the liver only by HSCs, and α-SMA, upregulated by activated HSCs, indicated that the isolated cells were indeed HSCs (Fig. 1A). To understand whether HSCs can act directly as APCs to induce Ag-specific proliferation of naive CD4$^+$ T cells, we enriched HSCs from the liver of a C57BL/6 mouse and cocultured these cells in combination with naive CD4$^+$ T cells from an OT-II transgenic mouse in the presence of OVA peptides 323–339. In the representative experiment shown, HSCs did not stimulate the proliferation of naive CD4$^+$ T cells, whereas splenic DCs strongly stimulated T cell proliferation (Fig. 1B). Thus, under these conditions, we found that HSCs were incapable of directly inducing proliferation in naive CD4$^+$ T cells in an Ag-specific manner.

HSCs influence DC-primed T cells to differentiate into Foxp3$^+$ Tregs

Because HSCs did not prime T cells directly in this system, we asked whether HSCs could influence the DC-induced differentiation of T cells. To this end, we isolated naive OT-II T cells and stimulated them with cognate Ag in the presence of HSCs, splenic DCs, or HSCs and splenic DCs combined. We detected minimal Foxp3 expression in OT-II T cells cultured with HSCs (3%) or DCs (4%) alone, or from the HSC+DC coculture (4%; Fig. 2A). The addition of exogenous TGF-β1 induced a significant fraction of the CD4$^+$ T cells derived from HSC+DC coculture to express Foxp3 (18% at 1 ng/ml), whereas no Foxp3 induction was observed in the absence of exogenous TGF-β1 (Fig. 2A). With the addition of exogenous TGF-β1, Foxp3 induction was observed in OT-II cells cultured with splenic DCs, but importantly, a significantly higher fraction of the OT-II T cells expressed Foxp3 (18%) when HSCs were also present. This demonstrated that HSCs had a positive influence on Treg development in this culture. To test

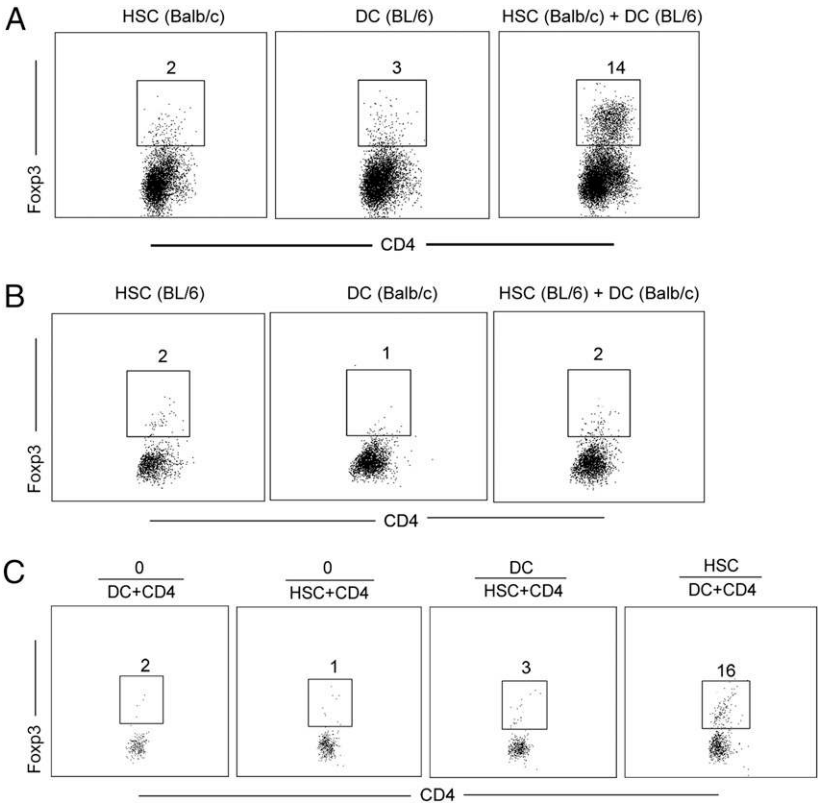

FIGURE 3. Ag presentation in the context of HSC activation induces Foxp3 expression. (**A**). Magnetic bead–isolated OT-II CD4$^+$CD62L$^+$ T cells were cultured with mismatched HSCs from BALB/c mice liver (HSC BALB/c) with OVA peptides 323–339 and TGF-β1. (**B**) Similar experiment as (A) except mismatched DCs were isolated from BALB/c mice spleen (DC BALB/c) and cocultured with naive CD4$^+$ T cells. The representative data shown were confirmed in three independent experiments. (**C**) Purified OT-II CD4$^+$CD62L$^+$ T cells were cocultured with splenic DCs and HSCs (either HSCs or DCs were separated by a transwell insert as indicated) for 90 h in the presence of OVA and TGF-β1. The representative data were confirmed in two independent experiments.

whether this observation would hold true for liver-resident $CD4^+$ T cells, which may respond differently to the stimulation conditions, we performed similar coculture experiments using intrahepatic OT-II T cells and assessed the HSC-mediated effects in these cultures. We found that the level of Foxp3 inductions for liver-resident $CD4^+$ T cells during priming was comparable with that observed for splenic $CD4^+$ T cells (Fig. 2B), and that this expression was also positively influenced by the presence of HSCs. Although the addition of a low concentration of TGF-β1 induced a significant fraction of the OT-II T cells derived from HSC+DC coculture to express Foxp3, DCs alone were only able to prime a small fraction of $Foxp3^+$ cells at very high TGF-β1 concentration (10 ng/ml; Fig. 2C).

In addition, we also analyzed the expression of key molecules associated with other major lineages of $CD4^+$ T cells: IFN-γ (TH1), IL-10 (TH2), and IL-17 (TH17). IFN-γ, IL-17, and IL-10 were measured after restimulation of cells cocultured with HSCs and DCs under the same conditions. We observed induction of IFN-γ expression indicative of TH1 cells only in OT-II cells cultured with DC, which was inhibited by the addition of TGF-β1 (Fig. 2D). IL-17 expression was minimal and expressed only by OT-II cells cultured with DCs in the highest TGF-β1 concentrations (data not shown). IL-10 production by OT-II cells was not detected under any conditions (data not shown). These findings demonstrated that although HSCs alone were not capable of directly priming naive $CD4^+$ T cells, they could induce Treg differentiation during priming by professional APC.

Treg induction by HSC is MHC independent

To confirm that HSCs do not function as APCs directly, we separated the Ag-presenting capacity of HSCs and DCs in the coculture system in two experiments. First, we isolated these

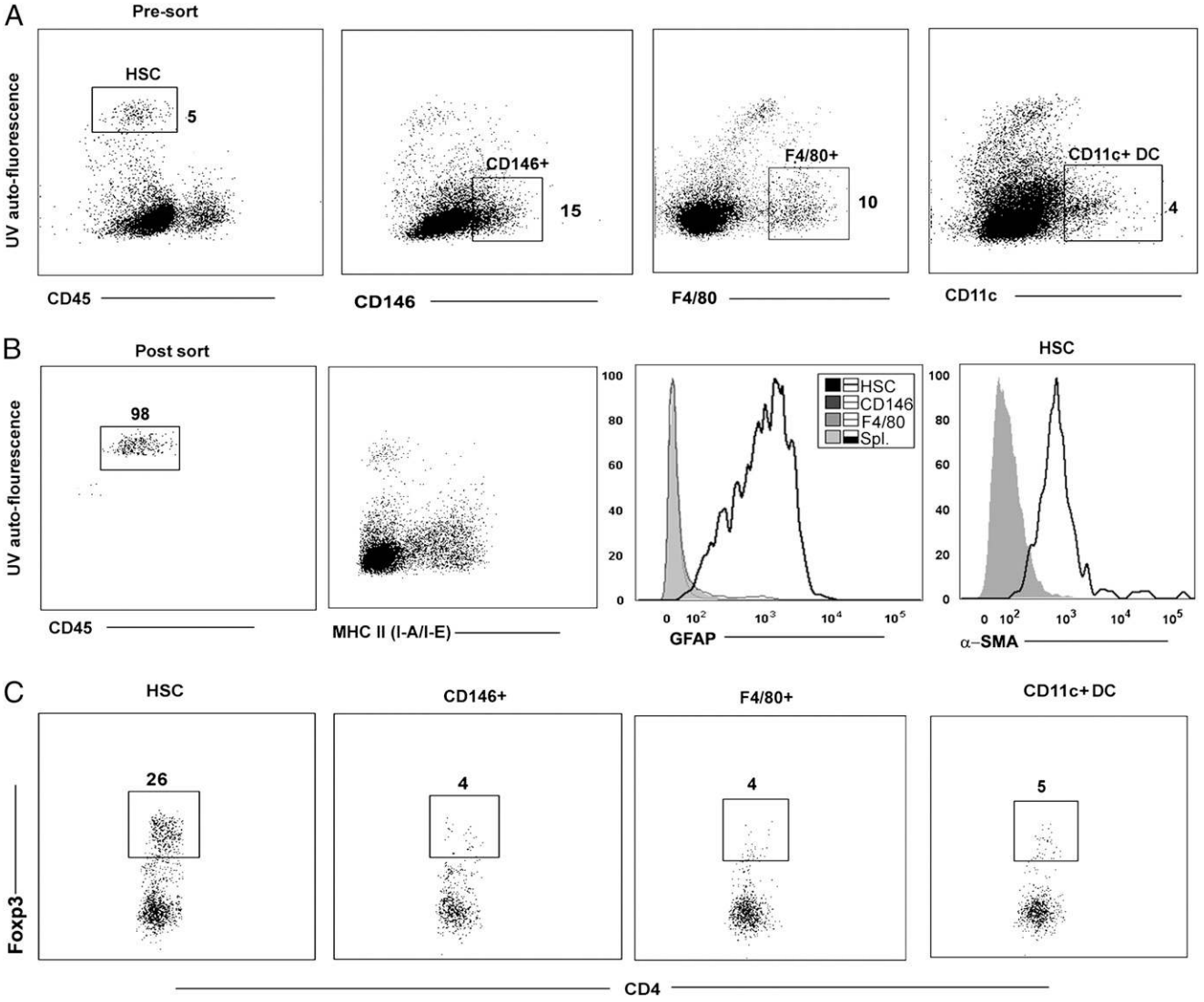

FIGURE 4. Isolation and purification of HSCs. Liver nonparenchymal cells from C57BL/6 mouse were isolated and enriched by density gradient separation. After enrichment, cells were stained with CD146 FITC, CD45 PerCP-Cy5.5, F4/80 allophycocyanin, or CD11c allophycocyanin Abs, and sorted on a FACSAria Cell Sorter II. (**A**) HSCs were identified as $UVAF^+$, $CD146^-$, and $CD45^-$, whereas liver cells, which are autofluorescence-negative ($UVAF^-$), lack CD45 but express CD146 marker, and represent LSECs (purity ~85%), were also identified. Other nonfluorescent liver cells that represent KCs ($UVAF^-$ $F4/80^+$, purity ~95) and liver DCs ($UVAF^-$ $CD11c^+$, purity ~88%) were also identified, respectively. (**B**) Purified HSCs and other nonfluorescent cells were further analyzed for their associated markers GFAP and α-SMA by intracellular staining. MHC class II expression on purified HSCs was also analyzed by flow cytometry. (**C**) Purified HSCs (2×10^4) and other liver nonparenchymal cells were cocultured with purified OT-II $CD4^+CD62L^+$ T cells (1×10^5) with splenic DCs (1×10^5) for 90 h in the presence of cognate Ag, OVA peptides 323–339 at 10 μg/ml, and TGF-β1 (1 ng/ml). Representative flow cytometry plots shown were confirmed in more than three independent experiments.

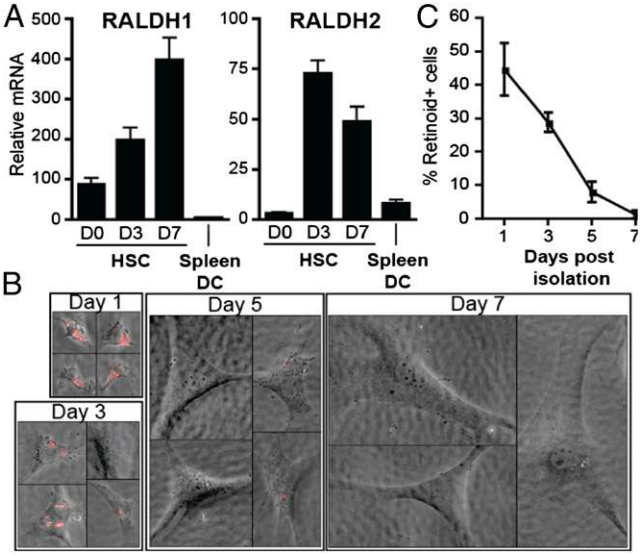

FIGURE 5. HSCs metabolize retinoids to form retinoic acid during activation. (**A**) Relative mRNA expression of RALDH1 and RALDH2 as measured in spleen DCs directly after isolation or HSCs directly after isolation or after culture for 3 or 7 d on nontissue culture–treated plastic to activate. Data shown are the average of three repeated measures and are representative of three independent experiments. Activated HSCs lose retinoid content as measured by UVAF in representative images (**B**), which is consistent in the average of 10 images/time point in more than three independent experiments (**C**).

populations from either BALB/c mice (I-A^d) or C57BL/6 mice (I-A^b) where only I-A^b–expressing APCs from C57BL/6 mice are capable of presenting OVA. Importantly, when HSCs from BALB/c mice and DCs from C57BL/6 mice were used in the coculture experiment, we observed Foxp3 expression despite being cultured with HSCs incapable of presenting cognate Ag (Fig. 3A), although, reciprocally, when only HSCs expressed I-A^b, proliferation and expression of Foxp3 was not observed (Fig. 3B). Second, we separated HSCs or DCs (both from C57BL/6 mice) from $CD4^+$ T cells using transwell inserts. If we separated DCs from the HSCs and $CD4^+$ T cell culture by transwell insert, we found no T cell priming, similar to HSCs and $CD4^+$ T cells alone. However, significant Foxp3 induction was observed when HSCs were separated from the DCs and $CD4^+$ T cell coculture (Fig. 3C). Together, these data confirm that HSCs can modify naive T cell priming by DCs and demonstrate that this influence is mediated by soluble factors.

Foxp3$^+$ Treg induction is specific to HSCs, not other liver-resident cells

The enrichment technique used earlier results in enrichment of HSCs, but not a purified population. To confirm that the phenomena observed earlier were due to HSCs and not a contaminating cell population, we purified HSCs after enrichment using FACS cell sorting based on vitamin A–mediated UVAF (35–37). HSCs were identified as $UVAF^+$ and lacking the expression of LSEC marker CD146 and hematopoietic cell marker CD45 (Fig. 4A). Other liver-resident APCs could also be identified, based on their expression of markers such as CD146 (LSEC), F4/80 (KC), and CD11c (DC; Fig. 4A). To confirm that the isolated $UVAF^+$ cells were HSCs, we measured expression of GFAP and α-SMA by flow cytometry and found that $UVAF^+$ HSCs expressed elevated levels of GFAP and α-SMA compared with other nonfluorescent liver cells and splenocytes (Fig. 4B). Importantly, the freshly isolated $UVAF^+$ HSCs did not express MHC class II, consistent with the observation that the HSCs cannot directly prime $CD4^+$ T cells (Fig. 4B). After the identification and phenotyping of HSCs and the various liver-resident APC populations, we tested the influence of these populations on Treg differentiation. To this end, we cultured the sorted HSCs, LSECs, KCs, and liver DCs with naive OT-II cells, splenic DCs, TGF-β, and cognate Ag as described earlier. Consistent with our previous observations, pure populations of $UVAF^+$ HSCs resulted in Foxp3 induction in the context of DC priming and TGF-β1, whereas none of the other liver-derived subsets were capable of inducing Foxp3 induction (Fig. 4C).

HSCs metabolize retinoids to form retinoic acid during activation

We found earlier that HSCs can influence DC-mediated naive $CD4^+$ T cell priming to induce Tregs through a soluble factor. ATRA signaling through RARα has been recently described to be important for the development of Tregs (20, 38–41). Because HSCs are the major storage cell for retinol (vitamin A), the metabolic precursor of ATRA (32), and this retinol is lost during the activation program (33), we hypothesized that HSCs produce ATRA, and that this ATRA promotes Treg differentiation during T cell priming. We therefore investigated the production of ATRA by HSCs in our system. We first measured the expression of the key enzymes in the generation of ATRA. Retinol is initially converted to retinaldehyde by retinol dehydrogenases, which is subsequently converted, in the rate-limiting and irreversible step, to ATRA by the retinaldehyde dehydrogenases RALDH1 and RALDH2 (ALDH1A1 and ALDH1A2, respectively). We isolated RNA from HSCs directly after isolation or after 3 or 7 d of plate activation, as well as from freshly isolated splenic DCs, and used sequence-specific primers to perform quantitative RT–PCR to measure the expression of the rate-limiting RALDH1 and RALDH2. We found that plate activation of HSCs, which mimics in vivo activation (32), induced expression of both RALDH1 and RALDH2 (Fig. 5A) compared with minimal expression in splenic DCs. We then measured cellular retinoid content qualitatively in plate-

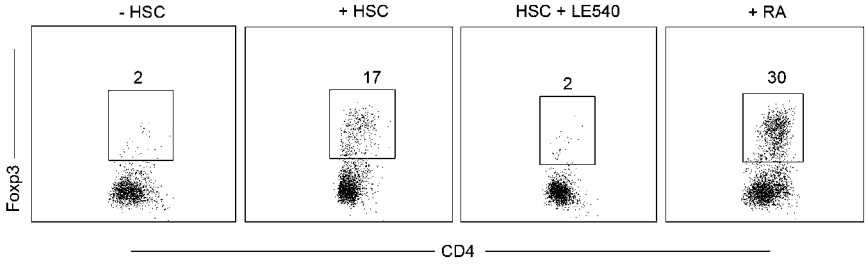

FIGURE 6. ATRA produced during HSC activation influences Treg priming. Magnetic bead–isolated OT-II $CD4^+CD62L^+$ T cells were cocultured with DCs with or without HSC, RA (100 nM), or LE540 (1 μM) at the indicated condition for 90 h with OVA peptides 323–339 (10 mg/ml) and TGF-β1 (1 ng/ml). Representative flow cytometry plots of Foxp3 expression in directly stained cells. HSCs and DCs were isolated from C57BL/6 mice liver and spleen, respectively. Data are representative of more than three independent experiments.

activated HSCs by UV fluorescence microscopy. UV-excited retinoid compounds exhibit a characteristic, rapidly fading fluorescence, detectable in isolated HSCs and in whole liver sections (42). We found that over the course of activation, HSCs greatly increase in size, differentiate into fibroblast-like cells, and lose the retinoid-specific UVAF (Fig. 5B, 5C). Importantly, the kinetics of RALDH1 and RALDH2 upregulation coincided with the loss of retinoid content in plate-activated HSCs as measured by UVAF.

ATRA production by HSCs during activation is essential for HSC-mediated Treg differentiation

To confirm that the ATRA produced by HSCs plays a role in Treg priming, we first confirmed that RA alone could induce Tregs during DC priming (Fig. 6). We then prevented interaction of RA with RARα during coculture of naive OT-II T cells with HSCs and DCs using the RARα-specific antagonist LE540. We found that the HSC-induced Foxp3 expression was strongly inhibited by LE540 (Fig. 6), supporting the hypothesis that HSC-derived ATRA is responsible for the HSC-specific induction of Treg differentiation.

Discussion

Basic mechanistic explanations for the observed tolerogenic nature of the liver have been elusive, despite decades of study. Although nonadaptive mechanisms such as constitutive immunosuppressive cytokine expression have been well documented (8), tolerance of the adaptive immune system in the liver is likely not global, but rather Ag-specific and mediated by Treg (6). However, it is not known how the liver generates Tregs. We hypothesized that a subset of hepatic cells may be directly or indirectly responsible for the differentiation of Tregs. Multiple cell types in the liver are reported to be capable of presenting Ag to $CD4^+$ T cells: liver DC (24, 25), KC (26, 27), LSEC (22), and HSC (23). Because HSCs are the major storage site for retinol, the metabolic precursor for ATRA, and produce the key Treg-polarizing molecules ATRA and TGF-β1 during activation, we focused our studies on the molecular interactions of HSCs with naive $CD4^+$ T cells. Using naive $CD4^+$ T cells derived from the OTII strain of TCR transgenic mice, we found that HSCs alone were unable to induce proliferation and differentiation, although in the presence of DC and low concentrations of exogenous TGF-β1, upregulation of Foxp3 was observed from the naive $CD4^+$ T cells during division. This finding confirms the findings by Ichikawa et al. (34) that HSCs indirectly prime DC-activated Tregs. This process was dependent on signaling through RARα and was associated with loss of HSC cellular retinoid content and concomitant upregulation of RALDH1 and RALDH2. Thus, we conclude that HSCs skew Th cell differentiation toward Tregs in a TGF-β1– and ATRA-dependent manner.

Treg conversion by HSCs, under the conditions reported in this study, is dependent on the addition of exogenous TGF-β, despite the previous descriptions of direct TGF-β production by HSC. This finding is likely due to the synchronicity of HSC activation in our coculture system, where all HSCs are at similar activation states. Thus, in our culture conditions, there does not exist a population of fully activated HSCs that have attained maximum TGF-β1 production (32). Such a population would be expected among an asynchronously activated HSC population observed in vivo, where HSCs would exist at various states of activation at any given time. In addition, other cells in the liver constitutively produce TGF-β1, including LSEC (43) and KC (44), indicating that TGF-β1 from other cells may act in concert with HSCs to induce Treg. We have established that ATRA is likely produced by HSCs in our coculture experiments by demonstrating a dependence of Foxp3 induction in naive T cells on RARα signaling, as well as showing that the retinoid content of HSCs is lost during in vitro activation concomitant with the induction of RALDH1 and RALDH2 expression. In addition, other groups have confirmed ATRA production by activated HSCs using HPLC (33). In vivo, other cytokines produced in the liver can also induce Treg priming and may act in concert with HSC-derived TGF-β1 and ATRA, such as LSEC- or KC-derived IL-10 (45, 46). In addition, KCs can induce Tregs through other mechanisms, such as galectin-9 expression (47).

These findings demonstrate a potential mechanism for the generation of Tregs within the liver environment and define a role for HSC:$CD4^+$ T cell interactions in liver immune tolerance. The experimental system described in this article involves in vitro activation of HSCs on plastic, modeling liver damage, and thus induction of the differentiation program reversibly converting these cells to myofibroblast-like cells. This differentiation is associated with the loss of cellular retinoid content, resulting in production of ATRA, critical to Foxp3 upregulation and Treg derivation. Because HSCs have been described as α-SMA–expressing activated cells during HCV infection (48, 49), in response to ischemia/reperfusion injury (50), and liver transplantation (51), this mechanism likely applies more to these scenarios involving liver damage than to steady-state oral tolerance, during which HSCs remain quiescent. Importantly, the finding that HSCs can influence Treg differentiation may explain the observations of increased Treg frequency in blood and liver during chronic HCV infection (15), which is implicated, on one hand, in impairment of the virus-specific T cell response (52, 53) and, on the other hand, limitation of liver fibrosis (54).

Overall, these findings provide insight into the phenomenon of immunological tolerance, the understanding of which is essential for modulation of this phenomenon in the clinical setting. For instance, augmentation of immunosuppression using HSC-targeted methods may enhance the acceptance of liver transplants and may potentially extend beyond the liver, where Tregs may be used to prevent autoimmunity, allergy, or rejection of nonliver transplants. In contrast, inhibition of HSC-mediated Treg derivation may allow the immune system to clear HBV and HCV infections during the acute phase, before the onset of fibrosis and cirrhosis. Although methods for the direct targeting of HSCs remain under development, this study provides an important advance in the understanding of liver immunobiology and a potential target for the treatment of immune-related liver diseases.

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Disclosures

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