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Hepatic Stellate Cells Function as Regulatory Bystanders

Shintaro Ichikawa,*† Daniel Mucida, † Aaron J. Tyznik,* Mitchell Kronenberg,* and Hilde Cheroutre*

Regulatory T cells (Tregs) contribute significantly to the tolerogenic nature of the liver. The mechanisms, however, underlying liver-associated Treg induction are still elusive. We recently identified the vitamin A metabolite, retinoic acid (RA), as a key controller that promotes TGF-β–dependent Foxp3+ Treg induction but inhibits TGF-β–driven Th17 differentiation. To investigate whether the RA producing hepatic stellate cells (HSC) are part of the liver tolerance mechanism, we investigated the ability of HSC to function as regulatory APC. Different from previous reports, we found that highly purified HSC did not express costimulatory molecules and only upregulated MHC class II after in vitro culture in the presence of exogenous IFN-γ. Consistent with an insufficient APC function, HSC failed to stimulate naive OT-II TCR transgenic CD4+ T cells and only moderately stimulated α-galactosylercamide–primed invariant NKT cells. In contrast, HSC functioned as regulatory bystanders and promoted enhanced Foxp3 induction by OT-II TCR transgenic T cells primed by spleen dendritic cells, whereas they greatly inhibited the Th17 differentiation. Furthermore, the regulatory bystander capacity of the HSC was completely dependent on their ability to produce RA. Our data thus suggest that HSC can function as regulatory bystanders, and therefore, by promoting Tregs and suppressing Th17 differentiation, they might represent key players in the mechanism that drives liver-induced tolerance. 

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Despite continuous exposure to bacterial components and dietary Ags (1), liver remains immune quiescent and is considered an immunosuppressive and tolerogenic organ (2). This is also demonstrated by the fact that liver grafts cause weak rejection and promote tolerance of cotransplanted tissues (3, 4). In addition, introduction of Ags via the portal vein leads to systemic tolerance (5). In contrast, its suppressive nature renders liver tissue highly susceptible to chronic viral infections, such as hepatitis virus B and C (6, 7). Foxp3-expressing regulatory T cells (Tregs) that suppress immune responses (8) are thought to play an important role in liver-mediated tolerance (9). Notably, increased Tregs are observed both in liver graft transplantations and chronic infections with hepatitis viruses, supporting a role for these cells in the immune suppression (10–13). Nevertheless, although the contribution of Tregs in mediating liver tolerance has been recognized (14–19), little is known about the mechanisms that drive liver-induced tolerance (10–13). Nevertheless, although the contribution of Tregs in mediating liver tolerance has been recognized (14–19), little is known about the mechanisms that drive liver-induced tolerance.

Abbreviations used in this article: ADH, alcohol dehydrogenase; DC, dendritic cell; DLD1, liver sinusoidal endothelial cell; HSC, hepatic stellate cell; iRFP, induced regulatory T cell; LSEC, liver sinusoidal endothelial cell; MHC II, MHC class II; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; Treg, regulatory T cell; VAD, vitamin A-deficient; VitA, vitamin A; WT, wild-type.

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Abbreviations used in this article: ADH, alcohol dehydrogenase; DC, dendritic cell; α-GalCer, α-galactosylercamide; HSC, hepatic stellate cell; iRFP, induced regulatory T cell; LSEC, liver sinusoidal endothelial cell; MHC II, MHC class II; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; Treg, regulatory T cell; VAD, vitamin A-deficient; VitA, vitamin A; WT, wild-type.

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and accumulation of Treg, which may lie at the basis of the tol-
erogenic nature of the liver.

Materials and Methods

Mice

C57BL/6, BALB/c, and OT-II TCR-transgenic (C57BL/6 background) mice were purchased from The Jackson Laboratory. VitA-deficient mice were generated in our animal facility by feeding a VitA-deficient diet starting at days 8–10 of gestation and continued after birth until 10 wk of age. All mice were maintained at the La Jolla Institute for Allergy and Immunology vivarium under specific pathogen-free conditions. Animal care and exper-
imentation were consistent with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Com-
mittee at the La Jolla Institute for Allergy and Immunology.

Abs and reagents

The following Abs were purchased from BD Biosciences, as purified or conjugated to FITC, PE, PerCP-Cy5.5, allophycocyanin, PE-Cy7, or biotin: anti-CD4 (L3T4), CD19 (1D3), CD24 (M1/69), CD25 (PC61), CD44 (IM7), CD45 (2D4.15), CD80 (16-10A1), CD86 (GL1), I-A/E (M5), TCR-β (RB6-8C5), TCR-γ (AB1) (BD Biosciences), TCR-β chain (H5-597), IL-2 (TC11-18H10), IFN-γ (XM1.2), desmin (RD301), GFAP (polyclonal), and isotype controls. Anti-mouse Fpox3 (FJK-16), F4/80 (BM8), biotin-
conjugated anti-mouse CD8 (53-6.7), CD25 (Bio7D4), CD11b (M1/70), CD1c (N418), CD62L (MEL-14), B2A2 (RA3-6B2), NK1.1 (PK136), Gr-1 (RB6-8C5) F4/80 (BM8), and TER-119 were purchased from ebi-
Science. Anti-CD146 (ME-9F1) was purchased from Biolegend. Alexa Fluor 488-conjugated anti-mouse IgG was purchased from Invitrogen. Anti-mouse CD16/32 used for Fc receptor blocking was produced in our labor-
atory.

Exogenous cytokines IL-6 (20 ng/ml), TGF-β1 (5 ng/ml), and IFN-γ (100 ng/ml) were from R&D Systems. MHC II–restricted OVA peptides

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Microscopic analysis of HSC

For immunofluorescence staining, sorted HSC were cultured in a cham-
ber slide for 2 d. Cells were fixed and permeabilized with a fixation/ permeabilization reagent (BD Biosciences) for 15 min. After washing
with permeabilization buffer, cells were stained with anti-desmin or anti-
GFAP for 20 min fixed in 4% Paraformaldehyde. Alexa Fluor 488 for 20 min. For phase-contrast microscopic analysis, cells were fixed with 4% formaldehyde for 10 min. Cells were mounted with ProLong Gold antifade
reagent with DAPI (Invitrogen) and analyzed with fluorescence micros-
copy (Zeiss).

In vitro culture

Sorted OVA-specific OT-II TCR transgenic T cells (1 × 10^7/well) were cultured with DC, HSC, or cultured HSC (2 × 10^7/well) in the presence of OVA peptide in 96-well plates for 4 d. According to the experimental conditions, IL-6, TGF-β1, RA, and LE540 were added in the concentration as described above. For the HSC coculture experiments, HSC or precul-
tured HSC (5 × 10^5) were added to the culture medium. To enrich HSC, HSC were cultured for 24 h to remove nonadherent cells, and T cells and DC were cultured with adherent HSC for 4 d in the same condition as described above.

FACS analysis of liver cells and cultured T cells

For surface cell analysis of HSC and DC, cells were stained with blocking Abs followed by anti-CD4 (for HSC) or anti-CD11c, CD80, CD86, or MHC II Ab. For intracellular staining with desmin or GFAP, cells were fixed and permeabilized with a Fixation/Permeabilization reagent (BD Biosciences) and stained with anti-desmin or GFAP followed by anti-
mouse IgG Alexa 488. Cultured T cells were stained for Foxp3 analysis or cytokine analysis. For Foxp3 staining, CD4^+ T cells were stained in-
tracellular using a Foxp3 staining kit (eBioscience). For cytokine staining,

Preparation and purification of HSC and LSEC

Mouse livers were perfused with Ca^2+ and Mg^2+-free HBSS and EGTA for 5 min followed by 0.4 mg/ml pronase (Roche) in HBSS and 0.5 mg/ml collagenase (Sigma-Aldrich) for 20 min. Digested tissues were mased

RNA isolation and real-time quantitative PCR

Sorted HSC, LSEC, or DC were washed with PBS and resuspended in TRizol (Invitrogen). The samples were frozen and kept at −80°C for later use. For RNA isolation, the suspended cells were homogenized, and RNA was separated from DNA by proteins with precipitation by chloroform and ex-
traction with isopropanol. cDNA was synthesized from the total RNA using the SuperScript II system (Bio-Rad) following the manufacturer’s instruction. Subsequently, cDNA was subjected to real-time PCR using SYBR green II (Roche) and the following primers: alcohol dehydrogenase (ADH) 1 forward; 5' AGACGCCGATGACGACTGG-3', ADH1 reverse; 5'GATCGTC-
GACGGCGGCAAGCACC-3'; ADH1 forward; 5'GTCGCTGAGTCCCTTGGCAGTAC-
CAAGAT-3'; ADH4 reverse; 5'GGTGATCTGGTGGACAGTTTTA-3'; ADH7 forward;
5'GCTTCTCATTGGAGGAGTAA-3', ADH7 reverse; 5'CACT-
TCCTACACACCCCTACATTT-3'; RALDH1 forward; 5'ATGTTGTTAGAGCAG-
GGCTTCTCTT-3', RALDH2 reverse; 5'CAGACATCCTTTGACATCCAA-
CGA-3'; RALDH2 forward; 5'GCTTGGTACGCTCTTCTTCT-3', RALDH2 reverse; 5'TCACCATCTTTCCTCCATTTCC-3', RALDH3 forward; 5'GAGACGTCTGACTGAAACTTCC-3', RALDH3 reverse; 5'TCAGGGGTTCTTCTCTCGAGT-3', desmin forward; 5'GAGGTTGGT-
TCAGGGGTTCTTCTCTCGAGT-3', desmin reverse; 5'CCTCCAGGGACAGTGC-
GAC-3'; GFAP forward; 5'GCCACCTAATACACGAA-3', GFAP reverse; 5'GCCGAGATGTGTTAGATC-3', L32 forward; 5'GAA-
ACTGCGGAAAACCCACA-3', L32 reverse; 5'GATTCTGCGGCTTAC-
CCT-3'. Gene expression was normalized by L32.
of iTreg, we first established conditions to highly purify the subset of HSC using FACS cell sorting. Although there are no reported HSC-specific surface markers, a CD45-negative but VitA-mediated autofluorescent-positive cell subset (34, 35) was readily detectable among the liver nonparenchymal cells of wild-type (WT) but not VitA-deficient (VAD) animals (Fig. 1A). These CD45-negative, autofluorescence-positive cells were then stained with anti-desmin and -GFAP Ab, both established intracellular markers for HSC (Fig. 1B). Consistent with this, CD45− but autofluorescent−sorted cells expressed >1000-fold higher desmin and 100-fold higher GFAP mRNA compared with nonfluorescent counterparts (Fig. 1C), and therefore the sorted subset contained >98% pure HSC (Fig. 1D). Purified HSC were cultured for 2 d and analyzed for morphology using phase-contrast and fluorescence microscopy. HSC displayed a characteristic star-like shape with oil droplets, and they stained positive for desmin and GFAP (Fig. 1E). These results indicate that a combination of the presence of VitA-mediated autofluorescence together with the absence of the cell-surface marker CD45 are specific determinants to mark and FACS purify the specific HSC subset derived from liver nonparenchymal cells.

**HSC do not express APC signature surface molecules**

To characterize if highly purified HSC express a phenotype characteristic of APC, we analyzed for the expression of MHC class I and II molecules as well as CD1d and typical costimulatory molecules, including CD80 and CD86 (Fig. 2). In contrast to DC, freshly isolated purified HSC did not express MHC II, CD80, or CD86 costimulatory molecules, including CD80 and CD86 (Fig. 2). Previous reports showed that upon freshly isolated purified HSC did not express MHC II, CD80, or CD86 (Fig. 2). In contrast to DC, class I and II molecules as well as CD1d and typical costimulatory characteristic of APC, we analyzed for the expression of MHC II, CD80, and CD86 (Fig. 2). In contrast to DC, class I and II molecules as well as CD1d and typical costimulatory characteristic of APC, we analyzed for the expression of MHC II, CD80, and CD86 (Fig. 2). In contrast to DC, class I and II molecules as well as CD1d and typical costimulatory characteristic of APC, we analyzed for the expression of MHC II, CD80, and CD86 (Fig. 2). In contrast to DC, class I and II molecules as well as CD1d and typical costimulatory characteristic of APC, we analyzed for the expression of MHC II, CD80, and CD86 (Fig. 2). In contrast to DC, class I and II molecules as well as CD1d and typical costimulatory characteristic of APC, we analyzed for the expression of MHC II, CD80, and CD86 (Fig. 2).

**HSC function as bystanders to enhance iTreg but inhibit Th17 differentiation**

The absence of MHC II expression and the inability of HSC to activate naive CD4 T cells render it very unlikely that HSC directly

**FIGURE 1.** Detection and purification of HSC. A, Liver nonparenchymal cells from WT or VAD mice were enriched by gradient separation, stained with anti-CD45 and CD146, and analyzed by FACS. VitA-derived autofluorescence was detected with the Pacific Blue channel. B, In addition to above staining, cells were further stained for intracellular desmin and GFAP and analyzed by FACS. Cells were gated on autofluorescence-positive, CD45-negative, or autofluorescence-negative populations. C, Autofluorescence-positive, CD45-negative cells were sorted and reanalyzed by FACS. D, mRNA was extracted from sorted LSEC, spleen DC, or cells as in C. GFAP and desmin mRNA expression was measured by quantitative PCR. E, Sorted autofluorescence-positive, CD45-negative cells (from BALB/c) were fixed and intracellularly stained for anti-GFAP or anti-desmin Ab, mounted with DAPI, and analyzed by phase-contrast or fluorescence microscopy. All experiments were repeated at least twice.
control the priming of CD4 T cells or the generation of iTreg. However, because HSC reside in the space of Disse, in close proximity of liver-resident APC, it is possible that HSC can indirectly regulate T cell responses as bystander cells. To determine whether HSC can cooperatively modulate the activation of T cells primed by professional APC, we cocultured naive OT-II T cells together with OVA peptide, DC, and freshly isolated and sort-purified HSC. Interestingly, freshly isolated HSC in the presence of DC and exogenous TGF-β greatly enhanced the generation of Foxp3+ T cells from 10.6 to 21% (Fig. 4A). Similarly, cultured HSC with or without IFN-γ also enhanced iTreg differentiation of DC-primed CD4 T cells (Fig. 4B). In contrast, HSC in the presence of DC and TGF-β plus IL-6 reduced Th17 differentiation from 22.6 to 15.4%, but still enhanced iTreg differentiation from 5.35 to 9.67% (Fig. 4C). These results suggest that, although HSC do not efficiently present Ags, they do exhibit regulatory bystander functions for both the generation of TGF-β-dependent iTreg as well as the inhibition of inflammatory Th17 cells’ differentiation.

**HSC promote iTreg induction and inhibit Th17 differentiation in an RA-dependent fashion**

The reciprocal effects on TGF-β-dependent differentiation of activated T cells are identical to those described for RA (25). To investigate whether purified HSC serve their bystander role in an RA-mediated fashion, we measured mRNA expression of ADH1, 4, and 7, which are enzymes involved in the metabolism of VitA to retinal, and RALDH1, 2, and 3, which metabolize retinal to RA (38). HSC expressed great amounts of ADH1 and RALDH1 mRNA (Fig. 5A). However, in contrast to mesenteric lymph node DC, which are also known to release RA and drive the iTreg differentiation and express high levels of RALDH2 (38), HSC expressed RALDH1. The activity of the enzyme to metabolize RA was further confirmed with the ALDEFLUORO assay (StemCell Technologies), which forms a substrate and exhibits fluorescence in response to enzyme activity (Fig. 5B).

To determine if the HSC-mediated increase of Foxp3 induction was RA dependent, we cultured naive OT-II T cells with DC and purified HSC in the presence of the RA receptor antagonist LE540 (27, 28). T cells cocultured with DC and HSC in the presence of LE540 did not show the increased iTreg differentiation, indicating that the HSC bystander effect was RA dependent (Fig. 5C). This was also confirmed using HSC from VitA-deficient (VAD) mice. Because VAD-derived HSC do not display autofluorescent, gradient-enriched HSC were used instead. Nevertheless, consistent with the absence of RA, enriched HSC from VAD mice failed to enhance Foxp3 induction on DC primed T cells (Fig. 5D). These data showed that the reciprocal effects of HSC on TGF-β-dependent T cell differentiation is mediated by their ability to release RA. Overall, the results show that the HSC do not function...

**FIGURE 2.** Phenotypic analyses of HSC. Spleen DC and HSC were stained with CD1d, CD80, CD86, MHC class I, and MHC II Abs. Cells were analyzed by FACS. For the analysis of cultured HSC, HSC was sorted by FACS and cultured for 7 d with or without IFN-γ before staining. Black line shows stained cells, and gray solid shows isotype control staining. All data are representative of at least two experiments.

**FIGURE 3.** HSC are not professional APC. A, Naive OT-II CD4 T cells were sorted and labeled with CFSE. T cells (1 × 10⁶/well) were cultured with 2 × 10⁶ purified DC, HSC, or activated HSC (cultured for 7 d with or without IFN-γ) from WT mice for 4 d in the presence of OVA peptide. T cell division was analyzed with FACS. Cells with more than one division were gated on. B, NKT cells were sorted from spleen as TCR- and NK1.1-positive cells. Sorted NKT cells (1 × 10⁴) were cultured with HSC or DC (1 × 10⁴) in round-bottom plates for 16–20 h in the presence of 50 ng/ml α-GalCer. To block CD1d engagement, anti-CD1d Ab (2 μg/ml) was added to the culture. After culture, IFN-γ concentration of cell-culture supernatant was measured with ELISA. Data show mean and SD of triplicate cultures. All data are representative of three independent experiments yielding similar results.
as regulatory APC, but that instead they harbor the unique ability to function as regulatory bystanders, which are able to control the reciprocal TGF-β-dependent differentiation of primed T cells in an RA-mediated fashion.

**Discussion**

Although the tolerogenic nature of the liver is well recognized, very little is known about the liver associated Treg induction, which is readily observed in liver allograft transplantations, hepatitis virus infections, or portal venous-induced tolerance (9–13). In this study, we aimed to elucidate the underlying mechanisms of liver Treg induction and accumulation. Based on the recently described reciprocal effects of RA that greatly promote TGF-β-driven iTreg generation and the unique ability of HSC to store and metabolize VitA, we reasoned that HSC could be the driving force for the enriched iTreg differentiation in the liver.

The density separation method widely used before to enrich HSC for the study of their phenotype and function has led to inconclusive and at times controversial data. To circumvent this problem, we established a new method to sort-purify HSC. We determined in this study that liver cells that are positive for VitA-specific autofluorescence but negative for the cell-surface molecule CD45 also display the typical HSC morphology as well as the intracellular expression of desmin and GFAP (Fig. 1 A,1 B,1 D). Therefore, these data indicate that the presence of autofluorescence together with the absence of CD45 staining are dependable markers to allow for FACS sort purification of HSC (Fig. 1 C). Furthermore, the absence of CD45 expression also indicates, in contrast to published data obtained with enriched HSC (39, 40), that HSC are of nonhematopoietic origin. Using sort-purified HSC, we show that HSC do not display an APC phenotype or function, and when analyzed ex vivo, they do not express detectable levels of MHC II or costimulatory molecules and fail to prime naive T cells in vitro (Figs. 2, 3 A). The discrepancy of our findings with those previously published underscores the importance of the degree of purity of the cells that are studied, and our study challenges previously made conclusions and demonstrates, using sort-purified cells, that HSC display neither an APC phenotype nor APC functions.

In contrast, freshly isolated HSC do express CD1, and IFN-γ–activated HSC do induce some MHC II expression that leads to the in vitro activation of NKT cells and conventional CD4 T cells, respectively; however, the observed activation is significantly weaker compared with that of DC (Fig. 3 B).
Nevertheless, although HSC function poorly as APC to activate CD4 T cells and therefore they are likely not directly involved in the biased generation of liver iTreg, our results in this study are the first, to our knowledge, to define a unique bystander role for HSC that enables them to greatly enhance iTreg differentiation of T cells primed by professional APC (Fig. 4A–C). These data suggest an indirect role for HSC to drive the immune suppressive and tolerogenic nature of the liver. In this context, it was shown indeed that cotransplanted HSC effectively protect islet allografts’ rejection (41).

The reciprocal effect of HSC bystander regulation to enhance TGFβ-dependent iTreg generation but inhibit TGFβ−dependent Th17 differentiation is identical to the recently defined RA-mediated immune regulation (25). This, together with the notion that HSC form the main storage place for VitA and the fact that Th17 differentiation is identical to the recently defined RA-expressing CD25+CD4+ regulatory T cells (Fig. 5), provide strong support to suggest that the regulatory bystander function of HSC is mediated via the release of RA during priming of T cells by professional APC. We directly confirmed this possibility by using either RA receptor inhibitor or VAD HSC and showed under such conditions that the regulatory bystander function of HSC was completely abolished (Fig. 5C, 5D).

Taken together, we show in this study that in contrast to published data, HSC do not directly stimulate T cells. However, in proximity of professional APC, HSC have the potential to cooperatively modulate T cell responses. Therefore, HCS function as regulatory bystanders, and they may contribute to the suppressive and tolerogenic features of the liver.

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Disclosures
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