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Hepatocytes Contribute to Immune Regulation in the Liver by Activation of the Notch Signaling Pathway in T Cells

Sven Burghardt,* Annette Erhardt,* Benjamin Claass,* Samuel Huber,†
Guido Adler,‡ Thomas Jacobs,§ Athena Chalaris,∥ Dirk Schmidt-Arras,∥ Stefan Rose-John,∥
Khali Karimi,* and Gisa Tiegs*

The “liver tolerance effect” has been attributed to a unique potential of liver-resident nonprofessional APCs including hepatocytes (HCs) to suppress T cell responses. The exact molecular mechanism of T cell suppression by liver APCs is still largely unknown. In mice, IL-10–dependent T cell suppression is observed after Th1-mediated hepatitis induced by Con A. In this study, we show that HCs, particularly those from regenerating livers of Con A–pretreated mice, induced a regulatory phenotype in naive CD4⁺ T cells in vitro. Using reporter mice, we observed that these T regulatory cells released substantial amounts of IL-10, produced IFN-γ, and failed to express Foxp3, but suppressed proliferation of responder T cells upon restimulation with anti-CD3 mAb. Hence, these regulatory cells feature a similar phenotype as the recently described IL-10–producing Th1 cells, which are generated upon activation of Notch signaling. Indeed, inhibition of γ-secretase and a disintegrin and metalloproteinase 17 but not a disintegrin and metalloproteinase 10, respectively, which blocked Notch activation, prevented IL-10 secretion. HCs from Con A–pretreated mice showed enhanced expression of the Notch ligand Jagged1 and significantly increased receptor density of Notch1 on CD4⁺ T cells. However, HCs from Con A–pretreated IFN regulatory factor 1⁻/⁻ mice, which cannot respond to IFN-γ, as well as those from IFN-γ⁻/⁻ mice failed to augment IL-10 production by CD4⁺ T cells. In conclusion, it seems that HCs fine-tune liver inflammation by upregulation of Jagged1 and activation of Notch signaling in Th1 cells. This mechanism might be of particular importance in the regenerating liver subsequent to Th1-mediated hepatitis.

Gut-derived microbial and food Ags continuously enter the liver via the portal vein without causing chronic inflammation. This is due to a unique microanatomical and immunological environment that leads to induction of immune tolerance. The “liver tolerance feature” was first described in 1969 by demonstrating long-term acceptance of liver allografts in pigs across the MHC mismatch without immunosuppression (1). Liver tolerance could mediate systemic tolerance since a non–liver graft was protected from rejection when it was cotransplanted with the liver allograft from the same donor. Moreover, frequent ineffective immune responses to local infections such as hepatitis B and C have also been ascribed to liver tolerance. Liver tolerance is not fully understood. However, the regulation of innate and adaptive immunity could be mediated by specialized liver-resident APCs, including liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), and tolerogenic “immature” dendritic cells (DCs) (reviewed in Refs. 2, 3).

Hepatocytes (HCs) are the parenchymal liver cell population and are mainly responsible for the metabolic function of the organ. Notably, they also function as APCs contributing to T cell activation and immune regulation in the liver. HCs interact and establish cell–cell contact with T lymphocytes through fenestrations in liver sinusoidal endothelial cells (4). HCs constitutively express MHC class I molecules, and MHC class II expression has been demonstrated in HCs in response to IFN-γ exposure or in patients with various forms of chronic liver disease (5). In addition to Ag-presenting molecules, HCs do express costimulatory molecules such as CD40 and CD80 (6, 7). Moreover, in diseases with chronically inflamed liver, for example, chronic hepatitis B and C or autoimmune hepatitis, they express the co-inhibitory molecule B7-H1 (8). Hence, HCs may contribute to the regulation of hepatic immune tolerance. For instance, purified HCs promote apoptosis in postactivated CD8⁺ T cells, which in turn leads to induction of functional tolerance in vitro (9). However, the role for HCs in CD4⁺ T cell anergy or CD4⁺ T cell tolerogenicity still remains to be clearly elucidated.

In an experimental model of Th1–mediated liver damage upon injection of the lectin Con A to mice, we have recently observed that acute IFN-γ– (10) and TNF-α–mediated (11) liver inflammation and liver injury are followed by long-term tolerance toward Con A restimulation, which was detectable as early as 8 d and persisted at least up to 42 d (12). This immunosuppressive status was characterized by resistance to induction of liver pathology, downmodulation of proinflammatory cytokines, and release of the anti-inflammatory cytokine IL-10, both locally and systemically. The requirement of IL-10 in Con A tolerance was proved with the help of anti–IL-10 receptor mAb or IL-10⁻/⁻ mice. Furthermore, IL-10


*Institute of Experimental Immunology and Hepatology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany; †Department of Immunology, Bernhard-Nocht Institute for Tropical Medicine, 2059 Hamburg, Germany; and ‡Institute of Biochemistry, University of Kiel, 24098 Kiel, Germany.

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Address correspondence and reprint requests to Prof. Gisa Tiegs, Institute of Experimental Immunology and Hepatology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, D-20246 Hamburg, Germany. E-mail address: g.tiegs@uke.de

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Abbreviations used in this article: ADAM, a disintegrin and metalloproteinase; DAPT, N-[N-(3,5-difluorophenacetyl)-L-ala]-γ-secretase inhibitor; DC, dendritic cell; GSI, γ-secretase inhibitor; HC, hepatocyte; IRF1, IFN regulatory factor 1; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; mRFP, monomeric red fluorescence protein; Treg, regulatory T cell; wt, wild-type.

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was found to be mainly produced by regulatory T cells (Tregs) and KCs (12). Nevertheless, others also provided evidence for T1 (Foxp3⁺) cells as a source of IL-10 (13).

Recently, an IL-10–producing Th1 cell subset that regulates immune responses independently of Tregs has been identified (14, 15). The IL-10 production by Th1 cells shown to be regulated by Notch signaling. Retroviral transduction of CD4⁺ T cells with different Notch isoforms resulted in a strong IL-10 production under Th1-polarizing conditions, and additionally the transduced cells coproduced IFN-γ and IL-2 (16). Delta-like 4, a Notch ligand that drives Th1 polarization (17, 18), is expressed in DCs and mediates IL-10 production, which could be abrogated by pharmacological inhibition of Notch signaling using a γ-secretase inhibitor (GSI) (16, 19). Notably, Notch cleavage upstream of γ-secretase is mediated by a disintegrin and metalloproteinase (ADAM) proteases, in particular by ADAM10 and ADAM17 (20). Interestingly, Notch/Jagged signaling is critical for liver regeneration in response to partial hepatectomy (21), hepatic fibrogenesis (22), and hepatocellular carcinoma (23). Moreover, mutations in this pathway have been described in liver diseases associated with the Alagille syndrome (24). In the immune system, the Notch ligands Jagged1 and Jagged2 have been attributed to Th2 and Treg conversion and suppression of autoimmune diseases (17). Moreover, upon administration of a Notch signaling inhibitor, GSI, to mice, the animals developed autoimmune hepatitis (25).

Liver inflammation is controlled by immune regulatory cytokines IL-10 and TGF-β (26, 27). Because liver-resident APCs are critically involved in the mechanisms of hepatic tolerogenicity, we sought to evaluate their contribution to the induction of immunoregulatory cells. In this study, we show that HCs contribute to induction of IL-10–producing CD4⁺ T cells through Jagged1–dependent Notch signaling. Furthermore, the induction of IL-10–producing CD4⁺ T cells by HCs could be pharmacologically blocked either by GSI or genetic/biochemical inhibition of ADAM17, suggesting that targeted modification of Notch ligand expression on HCs or interfering with Notch signaling might provide new tools to shift the level of tolerogenicity in the liver, for example, in hepatocarcinogenesis or autoimmune diseases.

Materials and Methods

Mice

Male IFN-γ⁻/⁻, IFN regulatory factor 1 (IRF1)⁻/⁻ (28), IL-10⁻/⁻, CD45.1, and double knock-in Foxp3-IRES-monomeric red fluorescence protein (mRFP) × IL-10 IRES GFP-enhanced reporter (tiger) mice, which enable detection of the well-defined and simultaneous expression of IL-10 (GFP) and Foxp3 (mRFP) (29), and sex/age-matched (8–10 wk old) C57BL/6 wild-type (wt) controls were bred in the animal facilities of the University Medical Center Hamburg–Eppendorf. Mice hypomorphic for the metalloprotease ADAM17 in all tissues (ADAM17ex/ex mice) have been described (30). Animals received humane care according to guidelines of the National Institutes of Health in Germany. Experiments were approved by the Institutional Review Board of Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz (Hamburg, Germany).

Animal treatments

Con A (8 mg/kg; Sigma-Aldrich, Munich, Germany) or saline was administered i.v.

Cell purification

Spleens were passed through 100-μm nylon meshes resulting in single-cell suspensions. T cells were isolated using a CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. To isolate splenic CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ responder T cells, splenocytes were sorted using a combination of MACS and FACSciBD FACSAria) as described previously to reach highly purified CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ responder T cells (∼98%). DCs were isolated using a CD11c Isolation Kit (Miltenyi Biotec) according to the manufacturer’s instructions.

Preparation of HCs

Primary HCs were isolated as described elsewhere. In brief, the liver was perfused with 10 ml Ca²⁺/Mg²⁺–free HBSS supplemented with 50 ml HEPES and 500 μM EGTA and digested with 0.004% Liberase (w/v) in situ, removed, and liver capsules were gently disrupted in PM medium (NaHCO₃–free HBSS with 10 m M HEPES and 0.2% BSA). Subsequently, single-cell suspensions were gently pressed through a 100-μm nylon mesh and the HCs were allowed to settle by gravity for 15 min. Parenchymal cells were separated from nonparenchymal cells by 90 Percoll gradient centrifugation.

In vitro coculture

For in vitro studies, HCs were isolated from wt or knockout mice 8 d upon saline or Con A pretreatment. HCs (1 × 10⁵) or splenic DCs (2 × 10⁵) were cocultured with 1 × 10⁶ splenic CD4⁺ responder T cells from wt mice or Foxp3-IRES-mRFP × tiger mice or ADAM17ex/ex mice in flat-bottom 96-well culture plates (Nalg Nunc International, Schwerte, Germany) in complete William’s medium (Life Technologies, Darmstadt, Germany) and complete RPMI 1640 medium (Life Technologies), respectively. Cells were cultured in the presence of plate-bound anti-CD3 mAb (5 μg/ml; BioLegend, San Diego, CA) for 48 h. When required, 20 μg/ml neutralizing anti–IFN-γ mAb was added to the culture medium. For transwell experiments, 24-well plates with 0.4-μm polycarbonate membrane inserts (Corning, Corning, NY) coated with anti-CD3 mAb (5 μg/ml) were used. Notch signaling was blocked by GSI N-[3-(3,5-difluorophenacetyl)-t- alanyl]-3-phenylglycine ε-buty1 ester (DAPT, 20 μM; D9542, Sigma-Aldrich) dissolved in DMSO (D2650; Sigma-Aldrich). Extracellular cleavage of Notch was blocked by the ADAM10 inhibitor G1254023X or the ADAM10/ ADAM17 inhibitor GW280264X (10μM) (31) that were dissolved in DMSO, respectively. The final concentration of DMSO in cocultures was 0.7%. DMSO (0.7%) was used as control as well.

Determination of cytokines

Sandwich ELISAs for IFN-γ and IL-10 were performed using Greiner Microlon 96-well high-binding flat-bottom microtiter plates (Greiner Bio-One, Frickenhausen, Germany). IL-10 Abs and IFN-γ DuoSet ELISA were purchased from BioLegend. Streptavidin-peroxidase and the tetramethylbenzidine substrate reagent set was purchased from BD Pharmingen (Heidelberg, Germany) and Roche Diagnostics (Mannheim, Germany), respectively, and used according to manufacturers’ instructions.

Flow cytometric analysis

Leukocytes or HCs were stained using a standard protocol including Fc blocking steps. The following anti-mouse mAbs were used: CD4-PE (RM4-5), CD4-allophycocyanin–H7 (GK1.5), CD25-PE (PC6I), IFN-γ–Alexa Fluor 700 (XM1G.1.2, all BD Pharmingen), CD4–BV605, CD45.1–PE-Cy7 (RM4-5), Notch1–allophycocyanin (HMN1-12), Notch3–PE (HMN3-133), H-2IAb-FITC (APF-6.120.1; all BioLegend, San Diego, CA). Data were recorded using the BD LSRII system and analyzed after gating out dead cells using BD FACSDiva software.

Real-time quantitative RT-PCR analysis

RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Darmstadt, Germany) or RNaseasy micro kit (Qiagen, Hilden, Germany) according to manufacturers’ instructions. Subsequently, total RNA was reversely transcribed following quantification by real-time RT-PCR using a Bio-Rad CFX96 real-time system and Absolute qPCR SYBR Green mix (Thermo Fisher, Schwerte, Germany). Primer pairs were used as described elsewhere. Relative mRNA levels were calculated after normalization to actin using the CFX96 Manager software.

Immunohistochemistry

Formalin-fixed paraffin-embedded liver tissue was used for immunohistochemical staining of Jagged1 according to the manufacturer’s instructions. The primary Ab to Jagged1 (1:250, ab7771; Abcam, Cambridge, U.K.) was incubated overnight at 4˚C. Ag masking solution (Vector Laboratories, Peterborough, U.K.) was used for Ag retrieval. Visualization was performed with ZymoChemPlus AP polymer kit (Zymed, Berlin, Germany) and Permanent AP Red kit (Zymed) according to the manufacturer’s instructions. The slides were counterstained with hematoxylin. Staining with the secondary Ab alone was used as negative control (data not shown).

Proliferation experiments

For suppression assays, CD4⁺CD25⁺ responder T cells were isolated from CD45.1 mice and labeled with cell proliferation dye eFluor 700 (eBio-
A previous study showed that HCs express MHC class II molecules in inflammatory conditions (5), allowing interaction of these cells with MHC class II–restricted CD4+ T cells. We initially examined HCs for MHC class II surface expression. Flow cytometry analysis revealed low but detectable levels of MHC class II molecule expression on the surface of HCs, which substantially increased in HCs isolated from mice 24 h after Con A injection. The increased expression of MHC class II on HCs from Con A–injected mice was reversible but increased again upon Con A restimulation (Supplemental Fig. 1). These data indicate that HCs bear the capacity to interact with CD4+ T cells. Next, we measured cytokine profile in supernatants obtained from a coculture system of HCs and CD4+ T cells. TCR stimulation of naive CD4+ T cells by anti-CD3 mAb alone produced only moderate levels of IL-2, IFN-γ, and IL-10. However, splenic DCs from both saline- and Con A–pretreated mice induced a significant release of IL-2 and IFN-γ from naive CD4+ T cells but they absolutely failed to induce IL-10 (Fig. 3A–C). This result emphasized the outstanding role for liver APCs in the induction of a tolerogenic milieu.

Liver DCs (32) as well as LSECs and hepatic stellate cells (33, 34) have been extensively studied for their Ag presentation capacity and induction of regulatory CD4+ T cells. Therefore, it was not unlikely that IL-10 induction by APCs is commonly observed in in vitro coculture systems. To verify the organ dependency of IL-10 production induced by HCs, we cultured splenic DCs with CD4+ T cells in the presence of anti-CD3 mAb. CD4+ T cells alone produced only moderate levels of IL-2, IFN-γ, and IL-10. However, splenic DCs from both saline- and Con A–pretreated mice induced a significant release of IL-2 and IFN-γ from naive CD4+ T cells but they absolutely failed to induce IL-10 (Fig. 3A–C). This result emphasized the outstanding role for liver APCs in the induction of a tolerogenic milieu.

**Results**

**HCs induce IL-10–producing CD4+ T cells that coproduce IFN-γ**

A previous study showed that HCs express MHC class II molecules in inflammatory conditions (5), allowing interaction of these cells with MHC class II–restricted CD4+ T cells. We initially examined HCs for MHC class II surface expression. Flow cytometry analysis revealed low but detectable levels of MHC class II molecule expression on the surface of HCs, which substantially increased in HCs isolated from mice 24 h after Con A injection. The increased expression of MHC class II on HCs from Con A–injected mice was reversible but increased again upon Con A restimulation (Supplemental Fig. 1). These data indicate that HCs bear the capacity to interact with CD4+ T cells. Next, we measured cytokine profile in supernatants obtained from a coculture system of HCs and CD4+ T cells. TCR stimulation of naive CD4+ T cells by anti-CD3 mAb alone produced only moderate levels of IL-2, IFN-γ, and IL-10. However, splenic DCs from both saline- and Con A–pretreated mice induced a significant release of IL-2 and IFN-γ from naive CD4+ T cells but they absolutely failed to induce IL-10 (Fig. 3A–C). This result emphasized the outstanding role for liver APCs in the induction of a tolerogenic milieu.

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**Statistical analysis**

Data are presented as mean values ± SEM. Results were analyzed using a Student t test or one-way ANOVA with a Bonferroni post hoc test. The p values < 0.05 were considered statistically significant.

**FIGURE 1.** HCs induce IL-10 production by CD4+ T cells. HCs were isolated from wt (A, C) or IL-10–deficient mice (B) 8 d after saline or Con A injection. Subsequently, HCs were cocultured with splenic CD4+ T cells from wt (A, B) or IL-10–deficient mice (C) and stimulated with anti-CD3 mAb. After 48 h, IL-10 release into supernatant was measured by ELISA. The representative data shown were confirmed in more than three independent experiments (n = 3). CD4+ T cells from Foxp3-IRES-mRFP × IL-10 IRES GFP–enhanced reporter (tiger) mice were analyzed by FACS to measure the frequency of IL-10–producing cells (D) upon cocultivation as described above. Cells were gated on CD4+ lymphocytes (n = 3). The representative data shown were confirmed in three independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
In general, APCs have been shown to interact with T cells via soluble factors and/or physical interaction (35–37). Therefore, we separated HCs and CD4+ T cells by transwells to characterize the mechanism of interaction. Fig. 3D clearly shows that HCs induced IL-10–producing CD4+ T cells in a cell–cell contact-dependent manner.

**FIGURE 2.** IL-10–producing CD4+ T cells coexpress IFN-γ and lack Foxp3 expression. CD4+ T cells from Foxp3-IRES-mRFP × IL-10 IRES GFP–enhanced reporter (tiger) mice were intracellularly stained and analyzed by flow cytometry to measure the frequency of IL-10+IFN-γ+ cells (A) and IL-10+Foxp3+ cells (B) upon cocultivation without HCs, with saline primed HCs, or with Con A–primed HCs. Cells were gated on CD47 lymphocytes (n = 3). The representative data shown were confirmed in two independent experiments.

**FIGURE 3.** Induction of IL-10 is restricted to liver-resident APCs and depends on cell–cell contact. IL-2 (A), IFN-γ (B), and IL-10 (C) production by CD4+ T cells was analyzed 48 h after cultivation without DCs (gray bars), with saline primed DCs (white bars), or with Con A–primed DCs (black bars). Cytokine production was measured by ELISA. The graphs represent the average of two independent experiments (n = 6). HCs, isolated either from Con A– or saline-treated mice, were cocultured with purified CD4+ T cells separated by transwell inserts in the presence of anti-CD3 mAb. Cocultures without transwell inserts were performed as a control. After 48 h, IL-10 (D) levels were determined by ELISA. The representative data shown were confirmed in two independent experiments (n = 3). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Th1 polarizing conditions modulate HC to promote IL-10 generation by CD4+ T cells

There is evidence that CD4+ T cells under Th1 polarizing conditions may produce IL-10 (16). The Th1 conditioning of HCs in vivo could be responsible for the increase of IL-10 release in our system, where CD4+ T cells were cocultivated with HCs from Con A–pretreated mice. To test this possibility, we used HCs from two different mice deficient in IFN-γ signaling, IRF1−/− and IFN-γ−/− mice. HCs from both saline- and Con A–pretreated IRF1−/− mice, which do not respond to IFN-γ, as well as those from IFN-γ−/− mice still induced IL-10 release in CD4+ T cells, but HCs from Con A–pretreated mice failed to augment IL-10 production by CD4+ T cells when compared with saline-pretreated controls (Fig. 4A). In contrast, in vitro neutralization of IFN-γ in the cocultures failed to block the enhanced IL-10 response induced by HCs isolated from Con A–pretreated mice (Fig. 4B), indicating that the in vivo Th1 polarizing condition induced by Con A pretreatment may modulate HCs, which is critical for increased expression of IL-10 in the CD4+ T cells.

Notch signaling regulates IL-10 production by CD4+ T cells

Notch signaling has been shown to be involved in regulation of IL-10 production in Th1 cells (16). Notch1 receptor expression was upregulated in CD4+ T cells in coculture with HCs from Con A–pretreated animals but not in coculture with HCs from saline-pretreated mice (Fig. 5A, 5B). No increase was observed in the levels of Notch3 expression (data not shown). Analysis of Notch ligand expression by HCs revealed a significant increase in Jagged1 mRNA levels in HCs isolated from Con A–pretreated mice (Fig. 5C) whereas mRNA levels of other Notch ligands, such as Jagged2 as well as Delta-like ligands 1, 3, and 4, were maintained at similar levels compared with HCs isolated from controls (data not shown). In line with previous findings (21), immunohistochemical analysis revealed localization of Jagged1 in HCs, which was increased in Con A–pretreated mice (Fig. 5D).

Changes in Notch receptor expression in cocultured CD4+ T cells and upregulation of its ligand prompted us to study the functional consequence of Notch signaling on IL-10 production in the coculture system. We used a GSI DAPT, which blocks Notch signaling by inhibition of proteolytic cleavage of the intracellular domain of Notch receptors, which functions as a transcription factor in the nucleus. Incubation of cells with GSI DAPT led to a significant inhibition of IL-10 production by CD4+ T cells, even in presence of HCs (Fig. 6A). Additionally, Con A–pretreated HCs were observed to induce the expression of the Notch specific target genes hes-1 and deltex-1 (18) in CD4+ T cells, which was significantly inhibited in the presence of GSI (Fig. 6B, 6C). Flow cytometric analysis revealed that the DAPT concentration used in the experiments was not toxic to CD4+ T cells (data not shown). Likewise, measurement of HC toxicity by lactate dehydrogenase release also revealed that DAPT is not toxic to these cells (data not shown).

Notch cleavage upstream of γ-secretase is mediated by ADAM proteases, including ADAM10 and ADAM17/TACE (20). To further investigate the involvement of Notch signaling in the IL-10 production by CD4+ T cells, we used two hydroxamate compounds, GW280264X, capable of blocking ADAM17 and ADAM10, and GI254023X, which preferentially blocks ADAM10 but not ADAM17 (31). As shown in Fig. 6D, GW280264X, but not GI254023X, reduced additional IL-10 release by CD4+ T cells in the presence of HCs from Con A–pretreated mice, indicating that ADAM17/TACE rather than ADAM10 mediated cleavage and activation of Notch in CD4+ T cells prone to differentiation into IL-10–producing CD4+ T cells. These findings were supported by further experiments employing CD4+ T cells isolated from ADAM17+/− mice (Fig. 6E). These mice express ∼5% of wt levels of ADAM17 in all tissues including T cells (30). Accordingly, GW280264X but not GI254023X inhibited Notch target gene expressions (Fig. 6F, 6G).

HC-primed CD4+ T cells suppress proliferation of naive CD4+ CD25− responder T cells

Having determined that there was an increase in IL-10 expression in CD4+ T cells in the coculture of HCs and CD4+ T cells, we wanted to assess whether this was associated with any suppressor activity indicative of functional regulatory cells. The HC-primed IL-10–producing cells were cocultured at different ratios with eFluor-labeled CD4+CD25− T cells under TCR stimulation using anti-CD3 mAb. HC-primed CD4+ T cells showed a significant (5.6-fold) increase in their ability to suppress proliferation of activated CD4+CD25− cells when compared with nonprimed controls (Fig. 7A, 7B). CD4+ T cells primed by either saline or Con A–pretreated HCs were able to inhibit T cell proliferation by ~80% at the highest ratio of HC-primed CD4+ T cells and responder cells (1:1). Given the evidence that IL-10 can be an important functional component of regulatory cells (38, 39), we investigated the role for IL-10 in the ability of HC-primed T cells to suppress T cell proliferation in vitro. Experiments in presence of anti-IL-10 mAb revealed that the superior capacity of HC-primed CD4+ T cells to suppress proliferation was independent of IL-10 in vitro (Fig. 7A, 7C). Additionally, we investigated whether Notch signaling is involved in further suppressor function of HC-primed T cells. As shown in Fig. 7D and 7E, presence of the GSI DAPT in HC-CD4+ T cell cocultures resulted in impairment of the
suppressive capacity of HC-primed CD4+ T cells allowing proliferation of eFluor-labeled CD4+CD25+ T cells in the suppression assay.

**Discussion**

In the present study we demonstrated that HCs, in particular those from mice with previous Con A–induced inflammatory liver damage, induce IL-10 secretion in naive CD4+ T cells in vitro that is accompanied by IFN-γ secretion. HC priming of CD4+ T cells was dependent on Notch receptor signaling in T cells and culminated in superior immunosuppressive abilities of these CD4+ T cells in vitro.

The liver is considered as an organ with unique tolerogenic capacities (40). Nonetheless, the exact cellular and molecular mechanisms for tolerance induction by the liver are still not fully understood. Functional investigations revealed outstanding qualities of liver nonprofessional and professional APCs, such as KCs, LSECs, hepatic stellate cells, and liver DCs to induce tolerogenic T cell responses (2, 3, 41, 42). Notably, HCs could also function as nonprofessional APCs because they express MHC class II under inflammatory conditions (Ref. 5 and this study) and interact and establish cell–cell contact with T lymphocytes contributing to T cell activation and immune regulation in the liver (4). Moreover, HCs can activate CD8+ T cells in a manner that leads to apoptosis of these cells (9). However, the role for HCs in induction of CD4+ T cell tolerogenicity and the underlying molecular interactions still remain to be clearly elucidated. In this study, we provide evidence that HCs modify naive splenic CD4+ T cells to IL-10–producing cells with immunoregulatory function in a Notch-dependent manner. This indicates a role for HCs in the tolerogenic ability of the liver.

We observed that HCs, in particular those from mice with previous Con A–induced inflammatory liver damage, induce high secretion of IL-10 accompanied by IFN-γ upon coculture with...
naive CD4+ T cells after TCR stimulation in vitro. Analogous to
our measurements of soluble IL-10 and IFN-γ in the coculture
supernatants, we mainly detected CD4+IL-10+IFN-γ+ cells on
a cellular level. Moreover, these CD4+ IL-10 producers were
largely negative for Foxp3, again featuring characteristics of IL-
10–producing Th1 cells (16). We demonstrated that the induction
of an IL-10+ Treg population was cell–cell contact-dependent and
was, importantly, specific for HCs and not a general characteristic
of all APCs, because splenic DCs from identically treated animals
did not induce IL-10 in naive CD4+ T cells upon coculture.

It was postulated recently that IL-10 secretion can be induced in
Th1 cells without diminishing IFN-γ secretion by Notch receptor
signaling (16). In accordance with that, we observed a significant
increase of Notch1 receptor density in CD4+ T cells in the pres-
ence of HCs from regenerating livers. Additionally, we determined
enhanced expression of the Notch ligand Jagged1 in HCs from
Con A–pretreated mice, which might be associated with an en-
hanced ability to induce IL-10 in cocultured CD4+ T cells. Our
findings indicated a functional role for Notch signaling in HC–
CD4+ T cell interactions in vitro, which was further supported by
inhibition of Notch proteolytic cleavage by DAPT or blockade of
ADAM proteases. DAPT inhibited HC-dependent IL-10 induction
in T cells and expression of the Notch target genes hes-1 and
deltex-1, which strongly suggested a functional relevance of Notch
signaling for IL-10 induction in HC–primed T cells. However,
inhibition of extracellular Notch cleavage by blocking ADAM10
and/or ADAM17 protease indicated that ADAM17/TACE rather
than ADAM10 mediated cleavage and activation of Notch re-
sion in the cocultured CD4+ T cells. Note that DAPT
completely inhibited IL-10 production, whereas inhibition or ge-
netic deletion of ADAM17 was less effective, which strongly sug-
gests that additional ADAMs might be involved in Notch cleavage.

Interestingly, partial hepatectomy results in enhanced Notch1
and Jagged1 expression in regenerating rat livers (21). Moreover,
IFN-γ was postulated to induce expression of Notch1 and Jagged1
in macrophages (43). Con A administration leads to very strong
IFN-γ–dependent inflammatory immune responses in livers of mice that culminate in severe hepatitis (10). Livers regenerate from Con A–induced hepatitis (44), and after regeneration mice are protected toward additional Con A stimuli by IL-10, which is most likely derived from Foxp3+ Tregs and Foxp32Tr1 cells (12, 13). We showed that HCs from Con A–pretreated IRF1- or IFN-γ–deficient mice failed to augment IL-10 production by CD4+ T cells. In contrast, IFN-γ blockade in the coculture could not abrogate enhanced IL-10 secretion. Therefore, it is conceivable that the previous “manipulation” of HCs in vivo under Th1-conditioned inflammation and/or subsequent liver regeneration contributes to conversion of HCs toward a more potent, Jagged1-expressing immunosuppressive liver-resident APC. Thus, our in vitro findings might have identified a new mechanism provided by HCs to possibly protect regenerated livers against inflammatory stimuli.

IL-10–secreting Th1 cells have been shown to limit immune pathology in the course of infection with intracellular pathogens, thereby controlling immune reactions independently of Tregs (14, 15). In this study, the regulatory phenotype of HC-primed IL-10–producing CD4+ T cells was emphasized by their superior ability to suppress anti-CD3 mAb-stimulated proliferation of naive CD4+ CD25+ T cells. However, the mechanism of T cell suppression by T cells with regulatory function in vivo and in vitro is still controversial (45). It seems to be well accepted that inhibition of T cell proliferation in vitro is cell–cell contact-dependent, but independent of soluble factors (46). This is in line with our finding that the suppressive capacity of HC-primed CD4+ T cells was independent of the origin of HCs, which suggests that at least in vitro suppression of T cell proliferation may be IL-10–independent. This was further supported by our observation that neutralization of IL-10 by specific Ab did not reverse T cell proliferation in vitro. Nonetheless, IL-10 released by HC-primed CD4+ T cells might be important to suppress T cell activation in vivo because several studies emphasized the outstanding T cell suppressive and anti-inflammatory properties of IL-10 in general (38, 39) as well as in the liver (12, 13). However, in contrast to IL-10 neutralization, inhibition of γ-secretase by DAPT significantly reversed proliferation of CD4+ responder T cells, indicating that the suppressive capacity of HC-primed CD4+ T cells is Notch signaling–dependent.

Taken together, our data indicate that HCs might contribute to the liver tolerogenic effect by induction of an IL-10–secreting T cell population with regulatory function in a Notch1 signaling–dependent manner. In response to Th1 conditions such as in the Con A model, the expression of MHC class II molecules in HCs is upregulated, and consequently HCs acquire a more potent immunosuppressive phenotype stimulating enhanced IL-10 secretion by CD4+ T cells owing to an increased expression of Notch li-
gand Jagged1 in the regeneration phase. IL-10 in turn is likely to downregulate the production of proinflammatory cytokines and may contribute to the effectiveness of the in vivo suppressive activity of CD4+ T cells and maintenance of the tolerance. Thus, blocking Notch signaling may represent a therapeutic option for situations where induction of liver tolerance could be detrimental as in hepatitis B virus and hepatitis C virus infections. Alternatively, Notch activation could also be targeted for immune modulation where immunological tolerance is appreciated, for example, in autoimmune diseases.

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Disclosures

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References


