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J Immunol published online 17 January 2014
http://www.jimmunol.org/content/early/2014/01/17/jimmunol.1301077

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/01/20/jimmunol.1301077.7.DCSupplemental

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Cleavage of the T Cell Protein Tyrosine Phosphatase by the Hepatitis C Virus Nonstructural 3/4A Protease Induces a Th1 to Th2 Shift Reversible by Ribavirin Therapy

Erwin Daniel Brenndörfer,* Anette Brass,* Juliane Karthe,† Gustaf Ahlén,* Johannes Georg Bode, † and Matti Sällberg*

Ribavirin has proven to be a key component of hepatitis C therapies both involving IFNs and new direct-acting antivirals. The hepatitis C virus–mediated interference with intrahepatic immunity by cleavage of mitochondrial antiviral signaling protein (MAVS) and T cell protein tyrosine phosphatase (TCPTP) suggests an avenue for compounds that may counteract these effects. We therefore studied the effects of ribavirin, with or without inhibition of the nonstructural (NS)3/4A protease, on intrahepatic immunity. The intrahepatic immunity of wild-type and NS3/4A-transgenic mice was determined by Western blot, ELISA, flow cytometry, and survival analysis. Various MAVS or TCPTP constructs were injected hydrodynamically to study their relevance. Ribavirin pretreatment was performed in mice expressing a functional or inhibited NS3/4A protease to analyze its effect on NS3/4A-mediated changes. Intrahepatic NS3/4A expression made mice resistant to TNF-α-induced liver damage and caused an alteration of the intrahepatic cytokine (IFN-γ and IL-10) and chemokine (CCL3, CCL17, CCL22, CXCL9, and CXCL11) profiles toward an anti-inflammatory state. Consistent with this, the number of intrahepatic Th1 cells and IFN-γ+ T cells in NS3/4A-transgenic mice decreased, whereas the amount of Th2 cells increased. These effects could be reversed by injection of uncleavable TCPTP but not uncleavable MAVS and were absent in a mouse expressing a nonfunctional NS3/4A protease. Importantly, the NS3/4A-mediated effects were reversed by ribavirin treatment. Thus, cleavage of TCPTP by NS3/4A induces a shift of the intrahepatic immune response toward a nonantiviral Th2-dominated immunity. These effects are reversed by ribavirin, supporting that ribavirin complements the effects of direct-acting antivirals as an immunomodulatory compound. The Journal of Immunology, 2014, 192: 000–000.

A bout 80% of the patients infected with hepatitis C virus (HCV) develop a chronic infection, which is connected with the development of liver fibrosis/cirrhosis, hepatocellular carcinoma, and/or liver failure (1). Hence, efficient HCV therapies aiming to prevent these complications through viral eradication are of high importance. The standard therapy for chronic hepatitis C consisting of pegylated IFN-α and ribavirin has been complemented recently by the HCV nonstructural (NS)3/4A protease inhibitors boceprevir and telaprevir, resulting in a better treatment response but enhanced side effects (2, 3). In both cases, the application of full-dose ribavirin was necessary to increase efficacy and decrease relapse rates in patients receiving the triple therapy (4, 5). Furthermore, clinical trials using newly developed direct-acting antivirals (DAAs) suggest that although IFN-α seems to be dispensable in future HCV regimens, ribavirin is necessary to prevent viral breakthrough and relapse (6). The addition of ribavirin to the combination of GS-9256, an NS3/4A protease inhibitor, and tegobuvir, an NS5B polymerase inhibitor, significantly enhanced antiviral activity of the DAAs by accelerating viral clearance and reducing the risk of resistance to the DAAs (7). Similarly, ribavirin significantly improved the treatment response in a study combining the NS3/4A protease inhibitor danoprevir and the NS5B polymerase inhibitor mericitabine (8). Although ribavirin on its own exerts a modest antiviral activity (9), its important role in HCV RNA decline and its capability to reduce the emergence of drug resistance suggest a distinct mechanism of action. Thus, the major role of ribavirin in HCV therapy may be immunomodulatory [e.g., by altering the Th1/Th2 ratio toward a more antiviral Th1-dominated immune response (10–12) and enhancing the IFN-induced gene expression (13–16)].

The HCV genome is coding for three structural (core, E1, and E2) and seven NS proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Apart from participating in the viral life cycle, some HCV proteins facilitate viral persistence by interfering with intrahepatic signal transduction (17). HCV NS3/4A in particular was shown to block innate immune pathways through proteolytic

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Received for publication April 24, 2013. Accepted for publication December 12, 2013.

This work was supported by the Swedish Cancer Society (to M.S.), the Swedish Research Council (to M.S.), the Stockholm County Council (to M.S.), and Vinnova (to M.S.); E.D.B. was supported by grants from the Swedish Society for Medical Research, the Ruth and Richard Julin Foundation, the Goljes Memorial Fund, the Lars Hierta Memorial Foundation, the Professor Nanna Svartz Fund, and the Karolinska Institutet. J.K. and J.G.B. were supported by grants from the Deutsche Forschungsgemeinschaft, in particular the collaborative research center 974 and the research group 729.

The supporters of this study had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DAA, direct-acting antiviral agent; p-galN, p-galactosaminase; HCV, hepatitis C virus; MAVS, mitochondrial antiviral signaling protein; NS, nonstructural; TCPTP, T cell protein tyrosine phosphatase; Tg, transgenic; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1301077
cleavage of mitochondrial antiviral signaling protein (MAVS) (18, 19) and Toll/IL-1R domain–containing adapter-inducing IFN-β (TRIF) (20). Furthermore, NS3/4A cleaves the T cell protein tyrosine phosphatase (TCPTP) (21, 22), which is known to regulate the Jak/STAT pathway (23–26), insulin signaling (27), and epidermal growth factor signaling (21, 28).

For spontaneous HCV clearance, a strong and sustained HCV-specific T cell response targeting multiple epitopes is necessary (29). HCV-specific T cells are attracted to the liver through the release of chemotactic chemokines and cytokines. Hence, HCV may avoid specific T cell migration to the liver during the early phase of infection by modulating the secretion of chemokines and cytokines in the liver (30, 31). Whereas Th1 immune responses are in general proinflammatory, induce cellular immunity, and are responsible for the killing of intracellular parasites, Th2 responses induce mainly humoral immunity and are often anti-inflammatory. Th1-type chemokines and cytokines are CCL3, CCL4, CCL5, CXCL9, CXCL10, CXCL11, and IFN-γ, whereas the chemokines CCL1, CCL11, CCL17, and CCL22 and the cytokines IL-4, IL-10, and IL-13 are connected with Th2-dominated immune responses (32).

We have previously reported that mice with liver-specific expression of NS3/4A are characterized by a decrease in intrahepatic IFN-γ production and that NS3/4A modulates the expression of the chemokines CXCL9 and CCL17. This results in a reduction of intrahepatic Th1 cells, which is paralleled by an increase in Th2 cells (33). In the current study, we have extended the analysis of the NS3/4A-mediated effects on intrahepatic immunity. Our data show that NS3/4A modulates the intrahepatic cytokine/chemokine expression and intrahepatic immune cell composition/functionality by cleaving TCPTP and that both ribavirin treatment and the inhibition of the NS3/4A protease can block the effects mediated by NS3/4A. Thus, this study helps us to understand the mechanisms used by HCV to modulate the host’s immune response to its own advantage. In addition, the study further elucidates the global mechanisms exerted by NS3/4A protease inhibition and helps to explain the immunomodulatory activity of the antiviral drug ribavirin.

Materials and Methods

Ethics statement

All mouse experiments have been approved by the ethical committee at Karolinska Institutet, and the mice were kept and monitored according to the official guidelines issued by Karolinska Institutet.

Mice

CBA × C57BL/6 mice transgenic (Tg) for full-length NS3/4A with functional protease (HCV genotype 1a) (34) and C57BL/6 mice Tg for full-length NS3/4A with defective protease (bearing the mutation Ile1074Val) (33) were bred and housed at the animal facility at Karolinska Institutet, Division of Comparative Medicine, Clinical Research Centre. All animals were analyzed for the presence of genomic NS3/4A as described (34). The respective wild-type (WT) mice were purchased from Charles River Laboratories (Sulzfeld, Germany).

Plasmids

The cDNA for murine TCPTP was generated from mRNA of NIH3T3 cells by reverse-transcriptase reaction and subcloned into the p3xFlag-CMV-7.1 expression vector (Sigma-Aldrich, St. Louis, MO). In the p3xFlag-TCPTPmut construct, point mutations were introduced into the two NS3/4A cleavage sites located at position aa 123/124 (rtvkeeask/wagwpdldre) and at position aa 216/217 (pdghpaavhe/aagiggrfgl) by PCR. The cysteine residue at position 123, the proline residue at position 128, the valine residue at position 213, and the serine residue at position 217 were all replaced by alanines, resulting in the sequences rtvkeeask/wagwpdldre and pdghpaavhe/aagiggrfgl. MAVS was previously cloned into pVAX1 (35). For the generation of pVAX1-MAVSmut, the cysteine on position 470 was mutated to alanine. All constructs were controlled by sequencing (MWG, Ebersberg, Germany).

Treatment of mice

The hydrodynamic injections were performed by injecting 1.8 ml Ringers solution containing 100 μg plasmid DNA i.v. in the tail vein within a period of 5–10 s. Liver injury was induced by giving LPS (5 ng/g mouse; Sigma-Aldrich) intraperitoneally in 100 μl PBS. Ribavirin (40 μg/g mouse; Sigma-Aldrich) was injected i.p. diluted in PBS at the time points described in the figure legends.

Samples from murine livers

Male 6–12-wk-old mice with liver-specific expression of NS3/4A and the corresponding WT mice were sacrificed by cervical dislocation. Their livers were perfused with PBS (pH 7.4, 4˚C) until they turned pale, whereafter they were excised. Whole-cell extracts were performed as described by Bredndörfer et al. (21).

ELISA

The mouse CCL3, CCL4, CCL5, CCL17, CCL22, and CXCL9 Quantikine ELISA Kits were purchased from R&D Systems (Minneapolis, MN). The mouse CXCL11 ELISA Kit from Abcam (Cambridge, U.K.), the mouse IFN-γ ELISA MAX SETs from BioLegend (San Diego, CA), and the HCV NS3 ELISA from BioFront Technologies (Tallahassee, FL). The ELISA analyses were performed according to the instructions of the manufacturers.

Isolation of intrahepatic lymphocytes

Male 6–12-wk-old NS3/4A-Tg mice and the corresponding WT mice were sacrificed by cervical dislocation. Then their livers were perfused with PBS (pH 7.4, 4˚C) until the livers turned pale. The perfused livers were excised, the gall bladder was removed, and the liver tissue was minced into small pieces with a surgical scissor. A single-cell suspension was made by pushing the liver with a syringe through a 70-μm cell strainer in a Petri dish. The cells were suspended in medium and centrifuged for 1 min with the off-brake setting at 850 × g and 22˚C. Then the supernatant was transferred to a new tube and centrifuged for 8 min with the high-brake setting at 480 × g and 22˚C. The resulting pellet was resuspended in 10 ml 37.5% Percoll (GE Healthcare, Uppsala, Sweden) and centrifuged for 30 min with the off-brake setting at 850 × g and 22˚C. Whereas the resulting pellet contains the intrahepatic immune cells, the remaining hepatocytes float to the top. To purify the lymphocytes, 1 ml RBC Lysis buffer (Sigma-Aldrich) was added. After incubation in room temperature for 1 min, 10 ml PBS was added followed by centrifugation for 5 min at 1200 rpm. The pellet was resuspended in cell medium, and the amount of lymphocytes was determined.

Flow cytometry

A total of 1 × 10^6 intrahepatic immune cells were stained for different surface markers and the intracellular cytokine IFN-γ using the following Abs: anti-mouse CD3 (clone 17A2) Pacific Blue, anti-mouse CD40 (clone DX5) R-PE, anti-mouse CD4 (clone RM4-5)allophycocyanin–Cy7, anti-mouse CD8a (clone 53-6.7) PerCP, anti-mouse IFN-γ (clone XMG1.2) FITC, anti-mouse CCR4 (clone 2G12) allophycocyanin, and anti-mouse CXCR3 (clone CXCR3-173) PE-Cy7 (all from BioLegend). LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) was used as a dead cell marker. Permeabilization of cells was performed using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Franklin Lakes, NJ). Cells were analyzed with the LSR Fortessa flow cytometer (BD Biosciences) and FlowJo software (version 9.3; Tree Star, Ashland, OR). Results were given as individual cytokines/chemokines produced by CD4+ T cells (gating strategy shown in Supplemental Fig. 1).

Western blot analysis

For immunoblot analysis, equal amounts of protein (60 μg) were subjected to SDS-PAGE (8–12% gel) and analyzed as described by Bredndörfer et al. (21). Abs against TCPTP and GAPDH were obtained from R&D Systems (Minneapolis, MN) and Biodesign (Saco) and the secondary Abs from DakoCytomation (Glostrup, Denmark).

Statistical evaluation

The quotient representing the control was set at one, and the second sample was calculated by dividing the value of the test sample with that of the control. Differences were considered significant at p < 0.05.

Statistically significant differences were determined by using the follow-
referring all controls to mean, which was set to one. The root mean square deviation is indicated. The significance was calculated using the Wilcoxon test.

Results

Intrahepatic NS3/4A expression shifts the intrahepatic cytokine and chemokine profiles toward an anti-inflammatory Th2-like state

We have previously demonstrated that liver-specific expression of NS3/4A in mice results in the modulation of the intrahepatic production of CCL17, CCL22, CXCL9, CXCL11, and IFN-γ (33). These mice are characterized by a reduction of the intrahepatic levels of the cytokine IFN-γ and the CXCR3 ligands CXCL9 and CXCL11, which positively regulate Th1 immunity. In contrast, intrahepatic levels of the CCR4 ligands CCL17 and CCL22, which trigger a Th2-dominated immune response, are increased in NS3/4A-Tg mice. To investigate the NS3/4A-induced alteration of the intrahepatic cytokine and chemokine profile more in detail, we analyzed the levels of the chemokines CCL3, CCL4, and CCL5 and of the cytokine IL-10 as well. Whereas the CCR5 ligands CCL3, CCL4, and CCL5 are predominantly associated with the Th1 response, IL-10 is an anti-inflammatory cytokine known to suppress the expression of Th1 cytokines and to be linked to Th2 immunity. As shown in Fig. 1A–C, the intrahepatic levels of CCL3 are significantly decreased in NS3/4A-Tg mice, whereas the levels of CCL4 and CCL5 are unchanged. Interestingly, the levels of IL-10 are significantly increased (Fig. 1D). The intrahepatic NS3 protein levels (1.54 ± 0.32 ng/mg liver protein) did not differ significantly between the groups of NS3/4A-Tg mice used for Fig. 1. Thus, NS3/4A expression induces a shift of the intrahepatic cytokine and chemokine profiles toward a nonantiviral Th2-dominated immunity.

Interestingly, intrahepatic expression of NS3/4A has also an effect on the systemic level of some of the studied cytokines and che-
mokines, because the serum levels of CCL17 and CCL22 were increased in NS3/4A-Tg mice, whereas the serum level of IFN-γ was decreased by NS3/4A expression (Supplemental Fig. 1). In contrast, the serum levels of CCL3, CXCL9, CXCL11, and IL-10 were on a similar level in both WT and NS3/4A-Tg mice (data not shown).

NS3/4A protease activity is necessary to modulate intrahepatic cytokine/chemokine expression and intrahepatic immune cell composition/functionality

To evaluate if the protease activity of NS3/4A is required for the NS3/4A-mediated modulation of intrahepatic CCL3 and IL-10 levels, NS3/4A-Tg mice expressing a protease-dead NS3/4A (mutNS3/4A-Tg

FIGURE 3. Role of MAVS and TCPTP in the NS3/4A-mediated modulation of intrahepatic chemokine levels. WT or NS3/4A-Tg mice (8–10 mice/group) were hydrodynamically injected with p3xFlag (control), p3xFlag-TCPTPwt (TCPTPwt), or p3xFlag-TCPTPmut (TCPTPmut) or with pVAX1 (control), pVAX1-MAVSwt (MAVSwt), and pVAX1-MAVSmut (MAVSmut). Three days later, mice were sacrificed, and their livers were perfused and used for the preparation of whole-cell extracts. The chemokine concentration in these samples was determined by using 500 μg protein/well in an ELISA specific for murine CCL3 (A), CCL17 (B), or CCL22 (C) or 200 μg protein/well in an ELISA specific for murine CXCL9 (D) or CXCL11 (E). For statistical analysis, the Wilcoxon test was used.
mice) were analyzed. As shown in Fig. 2A and 2B, mutNS3/4A-Tg mice exert neither the decrease in CCL3 expression nor the increase in IL-10 expression present in the livers of mice Tg for NS3/4A with functional protease activity.

We have previously reported that the intrahepatic expression of NS3/4A causes a decrease in the number of CD4+ T cells, IFN-γ-positive CD4+ T cells, and Th1 cells, which is paralleled by an increase in the number of Th2 cells (33). Because the protease activity of NS3/4A is needed for the NS3/4A-mediated modulation of the intrahepatic production of CCL3, CCL17, CCL22, CXCL9, CXCL11, IFN-γ, and IL-10 (Fig. 2A, 2B) (33), the NS3/4A-induced effects on the intrahepatic immune cell composition/ functionality were investigated in mutNS3/4A-Tg mice. Intriguingly, the amount of CD4+ T cells, IFN-γ-positive CD4+ T cells, Th1 cells (CXCR3+CD4+ T cells), and Th2 cells (CCR4+CD4+ T cells) in mutNS3/4A-Tg mice did not significantly differ from that observed in WT mice (Fig 2C–F, Supplemental Fig. 2). The intrahepatic NS3 protein levels were on a similar level in all groups of mice expressing NS3 (1.43 ± 0.27 ng/mg liver protein in the mice used for Fig. 2). Thus, the NS3/4A protease activity is necessary for the NS3/4A-mediated modulation of both the intrahepatic cytokine/chemokine expression and intrahepatic immune cell composition/functionality.

Additionally, the protease activity of NS3/4A is required for the NS3/4A-mediated effects on the serum levels of CCL17, CCL22, and IFN-γ (Supplemental Fig. 1).

**TCPTP is the NS3/4A protease substrate responsible for the NS3/4A-mediated effects on intrahepatic immunity**

To be able to analyze the role of the different NS3/4A substrates, constructs coding for WT MAVS and TCPTP or for MAVS and TCPTP bearing mutated NS3/4A cleavage sites were generated. We decided to focus on MAVS and TCPTP because the NS3/4A cleavage sites in MAVS and TCPTP are conserved in both their human and murine forms. In contrast, the NS3/4A cleavage site for TRIF described by Li et al. (20) is missing in the murine homolog, so that we (35) and others (36) were not able to detect NS3/4A-mediated cleavage of mouse TRIF.

NS3/4A-Tg and WT mice were treated by hydrodynamic injection of a control plasmid, a plasmid expressing WT MAVS or TCPTP, or a plasmid expressing mutated MAVS or TCPTP. Importantly, the NS3 protein levels measured in the livers of NS3/4A-Tg mice (1.48 ± 0.29 ng/mg liver protein) did not differ significantly among the groups used for Figs. 3–5. Hydrodynamic injection of the constructs coding for MAVS/TCPTP with mutated NS3/4A cleavage sites should compensate for the decreased intrahepatic levels of MAVS or TCPTP in NS3/4A-Tg mice. Interestingly, the injection of uncleavable TCPTP was able to reverse the NS3/4A-mediated effects on all chemokines (Fig 3) and cytokines (Fig. 4) analyzed. Moreover, even the hydrodynamic injection of constructs coding for WT TCPTP, resulting in an overexpression of TCPTP, was able to partly (CCL22, CXCL11, and IFN-γ) or fully (CCL3, CCL17, CXCL9, and IL-10) reverse the effects mediated by NS3/4A (Figs. 3, 4). In contrast, hydrodynamic injection of constructs coding for WT or mutated MAVS did not have any effect on the intrahepatic levels of CCL3, CCL17, CCL22, CXCL9, CXCL11, IFN-γ, or IL-10 in NS3/4A-Tg mice.

Most importantly, injection of uncleavable TCPTP was also able to reverse the NS3/4A-induced effects on the intrahepatic amount of CD4+ T cells, IFN-γ-positive CD4+ T cells, Th1 cells (CXCR3+CD4+ T cells), and Th2 cells (CCR4+CD4+ T cells) showing the functional relevance of TCPTP for intrahepatic immunity.

**FIGURE 4.** Role of MAVS and TCPTP for the NS3/4A-mediated modulation of intrahepatic cytokine levels. WT or NS3/4A-Tg mice (8–10 mice/group) were hydrodynamically injected with p3xFlag (control), p3xFlag-TCPTPwt (TCPTPwt), or p3xFlag-TCPTPmut (TCPTPmut) or with pVAX1 (control), pVAX1-MAVSwt (MAVSwt), and pVAX1-MAVSmut (MAVSmut). Three days later, mice were sacrificed, and their livers were perfused and used for the preparation of whole-cell extracts. The cytokine concentration in these samples was determined by using 200 μg protein/well in an ELISA specific for murine IFN-γ (A) or IL-10 (B). For statistical analysis, the Wilcoxon test was used.
immunity (Fig. 5A–D). In addition, injection of uncleavable TCPTP was able to reverse the NS3/4A-mediated resistance toward LPS/n-galN (Fig. 5E). Pretreatment with a construct coding for uncleavable TCPTP resulted in a significant decrease of the survival of LPS/n-galN–treated NS3/4A-Tg mice as compared with pretreatment with the empty control vector. Consequently, injection of the uncleavable TCPTP construct rendered NS3/4A-Tg mice equally sensitive to LPS/n-galN as WT mice.

Hence, these data suggest that the cleavage of TCPTP by NS3/4A plays a major role in the NS3/4A-mediated modulation of intrahepatic immunity.

**Pretreatment with ribavirin reverses the NS3/4A-mediated effects on intrahepatic immunity**

Several studies support an immunomodulatory role of ribavirin by strengthening Th1 immunity (10–12). This suggests that an important mechanism of ribavirin action against HCV may be the reversion of the NS3/4A-mediated alterations of the intrahepatic cytokine/chemokine profile. We investigated our hypothesis by treating NS3/4A-Tg and WT mice with ribavirin for up to 3 d with a new injection every 24 h and analyzing the intrahepatic levels of CCL3 through ELISA. As shown in Supplemental Fig. 3, 24 h of ribavirin treatment are able to partly reverse the NS3/4A-mediated effects on CCL3 expression, whereas 48 h of ribavirin treatment fully reconstitutes the levels of CCL3. Because a plateau in the level of CCL3 is formed after 48 h of treatment, the consecutive experiments were performed with 48 h of ribavirin treatment.

The analysis of all chemokines and cytokines modulated by NS3/4A revealed that ribavirin treatment has contrary effects on the intrahepatic levels of these chemokines and cytokines. Whereas ribavirin treatment is able to partly reverse the levels of CXCL9 and IFN-γ and fully reverse the levels of CCL3, CCL17, CXCL11, and IL-10 in the livers of NS3/4A-Tg mice, the levels of CCL22 in NS3/4A-Tg mice are decreased by ribavirin treatment even to a lower level than those in WT mice (Fig. 6).

**FIGURE 5.** The NS3/4A-mediated effects on the intrahepatic immune cell composition/functionality and susceptibility to LPS/n-galN are dependent on TCPTP cleavage. (A–D) WT or NS3/4A-Tg (NS3/4A) mice (8–10 mice/group) were hydrodynamically injected with either p3xFlag as control or p3xFlag-TCPTPmut (TCPTPmut). Three days later, mice were sacrificed, and their livers were perfused and used for the isolation of intrahepatic lymphocytes. The amount of CD4+ T cells (A), IFN-γ+CD4+ T cells (B), CXCR3+CD4+ T cells (C), and CCR4+CD4+ T cells (D) per gram of liver was determined by flow cytometry. For statistical analysis, the Wilcoxon test was used. (E) WT or NS3/4A-Tg mouse (10 mice/group) were hydrodynamically injected with either p3xFlag (control) or p3xFlag-TCPTPmut (TCPTPmut). Three days later, the mice were treated with 5 ng/g mouse LPS and 1 mg/g mouse n-galN. The survival of the mice was followed over a time period of 48 h. For statistical analysis, the Wilcoxon test was used. rel., Relative.
Finally, ribavirin treatment was also able to reverse the NS3/4A-mediated reduction in the intrahepatic amount of CD4+ T cells, IFN-γ–positive CD4+ T cells, Th1 cells (CXCR3+CD4+ T cells), and Th2 cells (CCR4+CD4+ T cells) (Fig. 7). These data suggest that ribavirin promotes a shift in the Th2/Th1 ratio toward a Th1 response and thus interferes with anti-inflammatory effects mediated by NS3/4A. In addition, ribavirin is also affecting the intrahepatic level of chemokines and cytokines in WT mice, but the effects of ribavirin in WT mice are much weaker than in NS3/4A-Tg mice (Fig. 6). The intrahepatic NS3 protein levels in NS3/4A-Tg mice (1.52 + 0.22 ng/mg liver protein) did not differ significantly between the groups used for Figs. 6 and 7.

Because ribavirin treatment does not affect the intrahepatic levels of TCPTP in NS3/4A-Tg mice (Supplemental Fig. 4), ribavirin does not seem to reverse the NS3/4A-mediated effects by inhibiting TCPTP cleavage.

**Discussion**

Recent clinical trials using new DAAs for the treatment of chronic hepatitis C have highlighted the central role of ribavirin in HCV therapy (3). Despite highly potent antiviral effects exerted by the combination of antivirals targeting two or more HCV proteins, ribavirin can significantly improve the treatment results (2, 37). Thus, it becomes difficult to argue for a direct antiviral effect of ribavirin as a key mechanism of action. In contrast, immunomodulatory effects mediated by ribavirin may be of high importance. Hence, the current study investigates the immunomodulatory effects of ribavirin and NS3/4A protease inhibition exerted on the intrahepatic immune response in greater detail.

The present study shows that NS3/4A TCPTP-dependently modulates the intrahepatic cytokine/chemokine expression, which results in a shift of the intrahepatic T cell profile toward a nonantiviral Th2-dominated immunity. Interestingly, inhibition of the NS3/4A
protease function causes a block of the NS3/4A-mediated effects. In addition, ribavirin treatment is also able to reverse most of the effects induced by NS3/4A. Thus, this study not only describes the mechanism of the NS3/4A-mediated interference with cellular immunity, but also demonstrates the effects of NS3/4A protease inhibition and ribavirin on the intrahepatic immune response.

We have recently demonstrated that in NS3/4A-Tg mice the intrahepatic levels of CXCL9, CXCL11, and IFN-γ are decreased, whereas the intrahepatic levels of CCL17 and CCL22 are increased (33). This study extends the previous findings by showing that the intrahepatic level of CCL3 is also decreased in NS3/4A-Tg mice. In contrast, the intrahepatic level of IL-10 is increased by NS3/4A expression. This suggests that the NS3/4A-mediated decrease in intrahepatic IFN-γ levels as well as intrahepatic Th1 and IFN-γ-positive CD4+ T cells (33) is induced by a downregulation of the expression of the chemokines CCL3, CXCL9, and CXCL11. The NS3/4A-mediated increase in the levels of CCL17, CCL22, and IL-10 further shifts the intrahepatic immune cell composition toward Th2 immunity, which is demonstrated by an increase in the number of Th2 cells. The NS3/4A-mediated decrease in the levels of CCL3, CXCL9, and CXCL11 may explain why the intrahepatic level of plasmacytoid dendritic cells, which express CCR5 and CXCR3, are decreased in NS3/4A-Tg mice (34). The discovery that intrahepatic NS3/4A expression results in a decrease in IFN-γ and an increase in CCL17 and CCL22 in the serum shows that NS3/4A produced in the liver has also an effect on the systemic level of cytokines and chemokines. This effect may contribute to the tolerance toward vaccination seen in NS3/4A-Tg mice (38).

The finding that the protease activity of NS3/4A is necessary for the NS3/4A-mediated resistance toward TNF-α–induced liver damage, the modulation of the intrahepatic chemokine and cytokine expression, and the alteration of the intrahepatic immune cell composition/functionality (33 and this study) prompted us to investigate which NS3/4A protease substrate is necessary for the effects induced by NS3/4A. In the last few years, several direct substrates of the NS3/4A protease such as MAVS (18, 19), TCPTP (21), and TRIF (20) were identified. Whereas NS3/4A cleavage sites in MAVS and TCPTP are conserved in both their human and murine forms, the NS3/4A cleavage site for TRIF is missing in the murine homolog. To analyze the relevance of MAVS, we mutated the cysteine on position 470 to alanine, which was shown to completely block the NS3/4A-mediated cleavage of MAVS (36). To analyze the role of TCPTP, the NS3/4A protease cleavage sites located at the positions 123/124 and 216/217 (21) were mutated. Interestingly, injection of a construct coding for uncleavable TCPTP but not for uncleavable MAVS was able to reverse the effects mediated by NS3/4A. That even the injection of a plasmid coding for WT TCPTP was able to at least partly reverse the effects mediated by NS3/4A is most likely due to the enhanced expression of TCPTP, which exceeds the catalytic capacity of the NS3/4A protease resulting in a compensation of TCPTP levels. That the injection of constructs coding for MAVS does not influence the NS3/4A-mediated effects can be explained by the fact that MAVS is mainly involved in the detection of double-stranded viral RNA, which is not produced in NS3/4A-Tg mice. In contrast, TCPTP is known to influence several pathways such as Akt (21, 27), MAPK (39), and Jak/STAT signaling (23–26). Which pathway TCPTP is using to modulate the expression of cytokines and chemokines is currently under investigation. That TCPTP plays an important role in the regulation of the immune response became clear when TCPTP-deficient mice were generated, which die 3–5 wk after birth be-
cause of systemic inflammation (40). Furthermore, single nucleotide polymorphisms in the gene coding for TCPTP have been linked to the development of rheumatoid arthritis and Crohn disease (41). The data reported in this study provide for the first time, to our knowledge, evidence that NS3/4A-dependent cleavage of the tyrosine phosphatase TCPTP plays an important role for the HCV-mediated misdirection of the immune response in hepatitis C and therefore importantly contributes to the subversion of antiviral immunity by HCV. The data further indicate that ribavirin is able to overcome the immunological aberrations resulting from NS3/4A-mediated cleavage of TCPTP.

The observation that both NS3/4A inhibition and ribavirin are able to reverse the global effects mediated by NS3/4A may have important implications for HCV therapy. The finding that inhibition of the NS3/4A protease function blocks the NS3/4A-induced effects on the intrahepatic immunity suggests that NS3/4A protease inhibitors such as the recently approved DAAs boceprevir and telaprevir may not only interfere with viral replication but also switch intrahepatic immunity to a more antiviral Th1-dominated immune response. In various clinical trials involving newly developed DAAs, ribavirin has been shown to be an essential component of HCV treatment therapy because regimens without ribavirin or with low-dose ribavirin were characterized by a significantly lower sustained virologic response, increased viral breakthrough, and higher relapse rates (2, 4, 5, 37). Thus, our data demonstrate that ribavirin treatment has a significant impact on the intrahepatic immune response, which is contrary to the Th2-supporting role of ribavirin in chronic hepatitis C. The data further indicate that ribavirin is able to reverse the global effects mediated by NS3/4A. This may have implications for HCV therapy because regimens without ribavirin or with low-dose ribavirin were characterized by a significantly lower sustained virologic response, increased viral breakthrough, and higher relapse rates (2, 4, 5, 37). Thus, our data demonstrate that ribavirin treatment has a significant impact on the intrahepatic immune response, which is contrary to the Th2-supporting role of ribavirin in chronic hepatitis C. The data further indicate that ribavirin is able to reverse the global effects mediated by NS3/4A.

In conclusion, the current study identifies TCPTP cleavage as a novel mechanism HCV is using to interfere with intrahepatic immunity and to promote viral persistence. This illustrates unknown global effects of protease inhibition and ribavirin, which may have an impact on future HCV therapy.

Disclosures
The authors have no financial conflicts of interest.

References

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Effect of intrahepatic NS3/4A expression on the serum level of CCL17, CCL22 and IFNγ. The serum of wild-type (WT), mut NS3/4A-Tg (Ile1073Ala) or wt NS3/4A-Tg mice (8-10 mice per group) was used to measure the concentration of CCL17, CCL22 and IFNγ. The chemokine or cytokine concentration in the serum was determined by using 50 µl serum/well in an ELISA specific for murine CCL17 or CCL22 or 25 µl serum/well in an ELISA specific for murine IFNγ. For statistical analysis the Wilcoxon test was used (NS = not significant).
Identification of intrahepatic CD4+ T cells. Intrahepatic lymphocytes were stained with a panel of antibodies including anti-mouse CD3 and anti-mouse CD49b to separate NK cells (CD49b+CD3-), NKT cells (CD49b+CD3+) and T cells (CD49b-CD3+) within the living single cell population. The T cell population was then further divided into CD8+ and CD4+ T cells and the Th1/Th2 profile of the CD4+ T cells analyzed.
Effect of ribavirin treatment on the intrahepatic level of CCL3. Wild-type (WT) or NS3/4A-Tg mice (8-10 mice per group) were treated with ribavirin (40 µg/g mouse) with either three doses 72h, 48h and 24h, two doses 48h and 24h or one dose 24h before sacrifice of the mice. Then their liver was perfused and used for the preparation of whole cell extracts. The CCL3 concentration in these samples was determined by using 500 µg protein/well in an ELISA specific for murine CCL3. For statistical analysis the Wilcoxon test was used.
Ribavirin treatment does not affect the intrahepatic levels of TCPTP. Wild-type (WT) or NS3/4A-Tg (NS3/4A) mice were treated with ribavirin (40 µg/g mouse) with 2 doses 48 and 24 hours before they were sacrificed. Then their liver was perfused, used for the preparation of whole cell extracts and analyzed by immunoblot using antibodies specific for TCPTP or GAPDH.