The Hepatitis C Virus Non-structural NS5A Protein Impairs Both the Innate and Adaptive Hepatic Immune Response in Vivo

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The role of hepatitis C virus (HCV) protein non-structural (NS) 5A in HCV-associated pathogenesis is still enigmatic. To investigate the in vivo role of NS5A for viral persistence and virus-associated pathogenesis a transgenic (Tg) mouse model was established. Mice with liver-targeted NS5A transgene expression were generated using the albumin promoter. Alterations in the hepatic immune response were determined by Western blot, infection by lymphocytic choriomeningitis virus (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection.

In vivo studies of NS5A-specific effects transgenic mice were generated with a liver-specific expression of NS5A. We used these mice to show that NS5A affects both the innate and the adaptive hepatic immunity.

**MATERIALS AND METHODS**

**Generation and Characterization of Transgenic Mice**—The NS5A gene derived from a genotype 1b isolate (GenBank™) was established. Mice with liver-targeted NS5A transgene expression were generated using the albumin promoter. Alterations in the hepatic immune response were determined by Western blot, infection by lymphocytic choriomeningitis virus (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection. Cytotoxic T lymphocyte (CTL) activity (LCMV), and using transient NS3/4A Tg mice generated by hydrodynamic injection.
accesion number D16435) was kindly provided by Dr. Kunitada Shimotohno (Kyoto University, Japan). The coding sequence for an N-terminal His6 tag and a C-terminal V5 epitope was fused to the NS5A coding sequence. The expres-
sion was performed under control of the mouse albumin prom-
tor/enhancer (16–19). The β-globin intron was included to
increase the expression level (16, 20). The poly(A) site was
taken from SV40 (18, 21). The NS5A expression cassette was
obtained by NotI/Xhol digestion of the vector pabN55A and
purified by preparative agarose gel electrophoresis. The frag-
ment was microinjected into FVB/N mouse embryos that were
implanted into pseudopregnant FVB/N mice. Cell culture
experiments comparing the untagged with the tagged construct
(data not shown) and previous work (17, 18) demonstrated that
the functionality of NS5A was not affected by fusion of the tags.
Chromosomal DNA was isolated as described and analyzed by
PCR using a β-globin intron-specific forward primer (5′-ctg-
gctggtatgcgtc-3′) and a NS5A-specific backward primer
(5′-atttgtagccgacctggaatg-3′). A tubulin-specific primer pair
was used as control. Glutamyl pyruvic transaminase (GPT)
serum levels were determined using the reflotron assay system
and 12 days the livers and spleens of the animals were homog-
ized, and infectious virus was titrated as PFU in liter cell
monolayer cultures (22).

Infection of Mice and Virus Titration—The plaque-purified
WE strain of LCMV was propagated in NCTC clone 929 L cells.
Mice were infected with 10^5 plaque-forming units (PFU); after 6
and hydrodynamic injection of mice using a codon optimized
complex assays were performed as described (26, 27) for details see additional supplemental information).

Statistical Analysis—Statistical analyses were performed using GraphPad Prism 4 software. The S.E. was used for deviation and for significance analysis Student’s test was used (p ≤ 0.05, *; p ≤ 0.01, **; p ≤ 0.001, ***). To calculate the significance of LCMV titer at day 12 p.i. the values for wild-type animals
were set as 10^2, the border of detection limit.

Isolation of Mouse Liver RNA Microarrays—RNA was iso-
lated from snap-frozen liver samples derived from three NS5A
transgenic 3-month-old mice and three corresponding sex- and
age-matched wild-type littermates as described (28). Each sam-
ple or reference RNA was transcribed into cDNA in the pres-
ence of Cy3- or Cy5-labeled dUTP, respectively. Hybridizations
were performed in the presence of an equal amount of refer-
ence RNA (Stratagene, La Jolla, CA) as described by Boldrick et
al. (29). The microarray results are based on three independent
experiments.

RT-PCR Analysis—For RT-PCR analysis RNA was isolated
from snap-frozen tissue using TRIzol (Invitrogen) according to
the instructions of the manufacturer. The INFβ, PKR, and
2,5′-OAS-specific RT-PCR was performed as described (30).
Serial dilutions of the corresponding cDNA served in every
PCR as a standard. In addition, induction of 2,5′-OAS and of
IFNβ was analyzed by Lightcycler PCR.

RESULTS

Generation of NS5A Transgenic Mice—To study the function of
NS5A in vivo, transgenic mice were established that express
the NS5A protein in the liver. The schematic structure of the
transgene is shown in Fig. 1a. Based on this construct, two inde-
pendent transgenic lines (01 and 03) were established that had
comparable expression levels of NS5A of the expected size
(Fig. 1b).

The expression of the transgene was analyzed by immuno-
fluorescence microscopy of liver sections derived from trans-
genic animals and the respective wild-type littermates using a
V5 tag-specific antisera. As expected for an ER-attached pro-
tein, the immunofluorescence microscopy for NS5A showed an
inhomogeneous cytoplasmatic staining with a concentration in
the perinuclear region, while the nucleus remains unstained
(Fig. 1c).

To verify the liver-specific expression, lysates derived from
liver and various other tissues (heart, kidney, spleen, and lung)
were analyzed by Western blotting using V5 tag-specific anti-
sera. The Western blot shows that NS5A was solely detectable
in the liver-derived lysates, confirming a liver-specific trans-
gene expression (Fig. 1d).

To study whether the expression of the transgene was
dependent on sex or age, liver-derived lysates from 3–12-
month-aged mice were analyzed by Western blotting. The blot
shows (i) that the transgene is constitutively expressed and (ii)

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pVAX1 were injected intravenously in the tail vein within
~5–10 s. 72-h posthydrodynamic injection, mice were sacri-
ficed, and liver tissue was taken for Western blot and immuno-
histochemistry experiments.
that the NS5A expression is slightly increased in male mice compared with the female animals (Fig. 1e).

To investigate if the transgenic NS5A is functional, we investigated the ability of NS5A to interact with Raf-1 and to mediate increase Raf-1 phosphorylation, similar to reports from cell culture (9). We could confirm an interaction of NS5A and Raf-1 with increased phosphorylation/activation of Raf-1 in the transgenic mice. Moreover, consistent with the cell culture experiments, the increased Raf-1 phosphorylation does not result in an activation of MEK/MAP2 kinase (see additional online information).

**Gene Expression Profile in the Liver of NS5A-expressing Transgenic Mice**

—To investigate the effect of NS5A on the gene expression profile, oligonucleotide arrays complementary to 15,000 genes were performed, comparing in independent experiments three transgenic mice to the corresponding three wild-type littermates. Genes that differed in expression levels by 1.5-fold or greater in each comparison, and in all experiments with a p value of less than 0.05 were considered. Based on these parameters, 37 genes could be identified as deregulated by the presence of NS5A. These are presented in Table 1, top and bottom and summarized in Fig. 2, a and b. The observed induction of Serpina 6, Inhibb, Ralbp1, and the repression of TXln, Pcdhga12, and Pdcd10 were confirmed by RT-PCR (data not shown). Thus, in these Tg mice, NS5A seems to affect the expression of several genes related to cell proliferation, differentiation, cell-cell contact, apoptosis, and protein degradation.

**NS5A Does Not Display a Transforming Potential in Vivo**—NS5A is considered as a factor possibly involved in the process of HCV-associated pathogenesis (13). To investigate whether the expression of NS5A is able to induce liver damage such as cirrhosis or HCC, the livers from 31 transgenic mice were analyzed histologically in comparison to their wild-type littermates.
TABLE 1
Deregulated gene expression in NS5A transgenic mice determined by microarray analysis

In the top section, genes more than 1.5-fold up-regulated in NS5A transgenic mice are shown. In the bottom section, genes more than 1.5-fold down-regulated in NS5A transgenic mice are shown.

| up-regulated genes                              | symbol | group | function                        | p     | x-fold |
|-------------------------------------------------|--------|-------|---------------------------------|-------|--------|
| Neurotrophin receptor associated death domain, mRNA (cDNA clone MGC:48230 IMAGE:1547029) | Nadd   | misc  | Opioid Receptor                 | 0.03  | 2.99   |
| Serine (or cysteine) peptidase inhibitor, clade A, member 6, mRNA (cDNA clone MGC:16492 IMAGE:4194164) | Serpna6 | misc  | Reproduction                    | 0.03  | 2.17   |
| Iminobeta-8 (imb8), mRNA                        | imb8   | misc  | Testicular peptide hormone      | 0.04  | 2.01   |
| Ral binding protein 1 (Rablp1), mRNA            | Rablp1 | contact | Cytoskeleton             | 0.03  | 1.94   |
| Mitogen-activated protein kinase 4, mRNA (cDNA clone MGC:73474 IMAGE:623544) | Mapk4 | protdif | Cell cycle                   | 0.04  | 1.81   |
| Glucagon-like peptide 1 receptor (Glp1r), mRNA  | Glp1r  | misc  | Glycogen metabolism            | 0.03  | 1.78   |
| Phospholipase A2, group VC (lysosomal, calcium-independent) (Pla2g4c), mRNA | Pla2g4c | protdif | Embryonal development         | 0.03  | 1.72   |
| Component of oligomeric golgi complex 2 (Cogs2), mRNA | Cogs2 | contact | Gog-vesicle transport         | 0.05  | 1.71   |
| Lymphotoxin-1-related membrane protein (Lmp), mRNA | Lmp   | protdif | Integral membrane protein at ER in lymphocytes (development?) | 0.04  | 1.65   |
| BCL2-like 11 (apoptosis facilitator), transcript variant 1, mRNA (cDNA clone MGC:28730 IMAGE:4469720) | Bcl211 | apotp | Apoptosis                    | 0.02  | 1.64   |
| Nuclear receptor coactivator 2 (Ncoa2), mRNA    | Ncoa2  | protdif | Glucocorticoid receptor interacting protein 1 (GRIP1) | 0.02  | 1.63   |
| Polydactyly disease 2 (Pd2), mRNA               | Pd2    | protdif | C2a+ channel, cell cycle       | 0.04  | 1.61   |
| Phosphatidylethanolamine-1-phosphate induced protein 1 (Pep1), mRNA | Pep1 | apotp | Apoptosis                   | 0.01  | 1.59   |
| Dual specificity phosphatases 9 (Dusp9), mRNA    | Dusp9  | protdif | Development                    | 0.04  | 1.58   |
| Synaptotagmin, mRNA (cDNA clone MGC:25678 IMAGE:4500116) | Synp | contact | Synaptic vesicles              | 0.01  | 1.58   |
| Glycophosphatidylinositol, mRNA (cDNA clone MGC:35819 IMAGE:3981538) | Gpiplb6 | protdif | (Proteolipid, myelin structure?) | 0.04  | 1.58   |
| X-ray repair complementing defective repair in Chinese hamster cells 3 (Xrc3c), mRNA | Xrc3c | misc  | DNA repair                    | 0.03  | 1.55   |
| NeuroNin, mRNA (cDNA clone MGC:5762 IMAGE:6490001) | Nexn3  | protdif | Neuron cell surface protein, signal transduction (C2a+) | 0.02  | 1.55   |
| PDZ domain containing RING finger, 3, mRNA (cDNA clone MGC:11955 IMAGE:3600693) | Pdzrn3 | ubiqu | Ubiquitination?                | 0.04  | 1.54   |
| Podothrin 7 (Pod7), mRNA                        | Pod7   | contact | Cell-cell recognition          | 0.05  | 1.54   |
| Isopenylcysteine carboxyl methyltransferase, mRNA (cDNA clone IMAGE:3155246) | Icmt | protdif | Carboxylation of case proteins, Ras-signalling | 0.03  | 1.53   |
| GIPC PDZ domain containing family, member 2, mRNA (cDNA clone MGC:18334 IMAGE:4286716) | Gipc2 | protdif | TGF beta Repl. III binding/signalling, membrane protein | 0.00  | 1.52   |

| down-regulated genes                              | symbol | group | function                        | p     | x-fold |
|--------------------------------------------------|--------|-------|---------------------------------|-------|--------|
| MOR mRNA for mu opioid receptor                  | Oprm1  | misc  | Opioid receptor                 | 0.03  | -1.50  |
| Mitogen-activated protein kinase kinase, kinase 4 (Mapk4k4), mRNA | Mapk4k4 | protdif | TNFα-Ly6Pathway               | 0.04  | -1.51  |
| Cadherin 6 (Cdh6), mRNA                          | Cdh6   | contact | Cell-Cell adhesion            | 0.05  | -1.53  |
| Ras and Rhinoceros 2 (Rho2), mRNA                | Rho2   | protdif | Endocytosis, Ras-sign??        | 0.04  | -1.53  |
| Stat1n, c-mycelin binding protein 3 (Smyc3), mRNA | Smyc3 | protdif | C-mycelin signalling           | 0.02  | -1.54  |
| Aldihyde dehydrogenase family 3, subfamily a2, mRNA (cDNA clone MGC:6055 IMAGE:3460119) | Alox3a2 | misc  | Aldihyde metabolism           | 0.00  | -1.55  |
| Myelin transcription factor 1-like (Myl1), mRNA   | Myl1   | protdif | Neurons, development and differentiation | 0.00  | -1.60  |
| Myocilin, mRNA (cDNA clone IMAGE:5846002)        | 4729655J9R4 | contact | Motor protein                  | 0.04  | -1.61  |
| Cdc42 GTPase-activating protein (Cdc4p), mRNA     | Cdc4p  | protdif | Linker between MAPK- and Rac1. Cytoskeleton | 0.00  | -1.66  |
| Ubiquitin specific peptidase 24, mRNA (cDNA clone MGC:41753 IMAGE:1381175) | Usp24 | ubiqu | Protein degradation?           | 0.01  | -1.81  |
| F-box protein 10, mRNA (cDNA clone MGC:107732 IMAGE:4636464) | Fbox10 | ubiqu | Protein ubiquitination        | 0.00  | -1.84  |
| Tawlin alpha (Tva), mRNA                         | Tva    | contact | Vesicle transport              | 0.05  | -1.85  |
| Podothrin gamma subfamily A, 10, mRNA (cDNA clone MGC:40408 IMAGE:5400956) | Podothrc10 | contact | Cell-cell recognition / adhesion | 0.00  | -1.87  |
| HECT domain containing 1, mRNA (cDNA clone IMAGE:3709271) | Hectd1 | ubiqu | Protein ubiquitination        | 0.00  | -1.89  |
| Programmed cell death 10, mRNA (cDNA clone MGC:10291 IMAGE:4022513) | Pced10 | apotp | Apoptosis?                    | 0.04  | -1.99  |
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Neither in the liver nor in any other organ, could tumors be detected even in the case of mice that were kept up to an age of 17 months. Besides the absence of tumors, there were no other pathological changes detectable such as neoplastic or preneoplastic changes as investigated by histological analysis based on HE and PAS staining (data not shown). Additionally there was no difference in markers for cell proliferation, such as PCNA, and ongoing cell transformation such as altered glycosylation pattern of the cell surface (investigated by lectin staining of paraffin sections) between NS5A transgenic mice and corresponding wild-type littersmates. Consistent with previous data (31), these data indicate that at least on the genetic background of FVB/N mice, NS5A per se is not sufficient to induce any pathological changes in liver in vivo.

NS5A Impairs Antiviral Response—To investigate the potential role of NS5A in the establishment of a persistent viral infections in vivo, 12 wild-type and 12 NS5A transgenic mice were infected with 10⁵ PFU LCMV. Six Tg and wild-type animals were sacrificed at days 3, 6 (peak of LCMV infection) 9, or 12 days postinfection, and the LCMV titers in the liver and in the spleen were determined. At days 3 and 6, there was a slightly elevated viral titer in the liver and in the spleen of the transgenic animals. However, at days 9 and 12 a significantly higher viral titer was found in the Tg mice in both the liver and in the spleen compared with the wild-type mice (Fig. 3a). The minor difference in viral titers seen at days 3 and 6 and the major differences seen at days 9 and 12 indicate that virus elimination is impaired by NS5A, but not the susceptibility to infection.

LCMV infection triggers a potent CTL response causing rapid viral clearance around day 10 postinfection (32, 33) and that ultimately leads to severe liver damage. Therefore, the prolonged infection in the transgensics should be associated with ongoing release of liver-specific enzymes. Serum levels of GPT levels were determined in infected animals at days 3, 6, 9, and 12. Fully consistent with the impaired elimination of LCMV in the transgenic animals, in the sera derived from the NS5A transgenic mice, significantly lower GPT levels were found for days 6 and 9 and significantly higher GPT levels were found for day 12 (Fig. 3b). This is in accordance to the persistent elevated amount of T cells in the liver of the NS5A transgenics after 12 days. In wt mice, a reduction in the amount of T cells can be observed after 12 days. T cells were visualized by a CD3-specific antiserum (Fig. 3c). In addition, HE staining of liver tissues from the different time points revealed in the case of the transgenics 12 days after infection, a persistant increased infiltration indicating an ongoing immune response in the case of the NS5A transgenics compared with the wild-type mice (Fig. 3d). Taken together, these data indicate that clearance of LCMV is impaired in NS5A transgenic animals.

NS5A Impairs Interferon Immune Response—To test whether an inhibited innate immune response contributes to the delayed virus elimination in the NS5A-Tg mice we analyzed the NS5A–PKR interaction. In cell culture experiments, it has been shown that NS5A (HCV genotype 1b) directly binds to PKR, thereby inhibiting PKR activity (15). This is important since PKR is involved in the double-stranded RNA-dependent induction of IFNβ, PKR, and 2′,5′-OAS are induced subsequently by interferon α/β (34). Co-immunoprecipitation experiments of liver lysates demonstrated that PKR indeed bound NS5A also in vivo (Fig. 4a). To study the effect of NS5A on the IFN response in greater detail, we analyzed the induction of IFNβ, PKR, and 2′,5′-OAS by RT-PCR and Western blotting at day 6 after infection. RT-PCR analysis showed a strong induction of IFNβ, 2′,5′-OAS and PKR in wild-type mice, and these were all inhibited in the NS5A-Tg mice (Fig. 4b). For IFNβ and 2′,5′-OAS, this was confirmed by Lightcycler PCR (Fig. 4d). Also, Western blotting using 2′,5′-OAS- or PKR-specific antisera showed that the induction of proteins was impaired in NS5A-Tg (Fig. 4c). Taken together, these results suggest that NS5A has a strong inhibitory effect on the innate immune response that contributes to impaired virus elimination.

NS5A Interferes with CTL Response—The difference in the viral titers between infected NS5A-Tg and wild-type mice was most pronounced at day 12. Taken into consideration that T-cell response peaks after the first week postinfection (33), an effect of NS5A on the adaptive immune response may be postulated. To test this possible interference of NS5A with the CTL response, we made the NS5A-Tg mice transiently transgenic for NS33/4A proteins by hydrodynamic injection. In this model, NS3/4A-expressing liver cells are effectively eliminated by vaccine-primed CTLs (24), whereby an impaired clearance indicates an effect restricted to CTL function. Thus, NS5A-Tg and wild-type mice were vaccinated with coNS3/4A-DNA, and 13 days later they were all given a hydrodynamic injection of the same coNS3/4A plasmid (26). Three days later, the mice were sacrificed, and the presence of NS3-expressing cells was determined by immunohistochemistry and Western blot (Fig. 5a). This revealed that most NS3/4A-expressing cells were elimi-
nated in the wild-type mice, whereas NS5A-Tg mice had a significantly higher number of NS3-positive hepatocytes. This was confirmed by Western blot analysis of liver lysates, where we also noted that mice with a high level expression of NS5A had the highest levels of NS3 (Fig. 5c). This suggests that a certain amount of NS5A is needed to protect NS3/4A-positive cells from CTL-mediated elimination (Fig. 5c).

To exclude that NS5A affects the expression of NS3/4A, pVaxNS3/4a was cotransfected with palbNS5A or with the empty control vector (palb) into HuH7.5 cells. The Western blot analysis revealed that the presence or absence of NS5A does not affect the amount of NS3 (Fig. 5f). Comparable results were obtained after hydrodynamic injection of pVaxNS3/4a in wt and NS5A transgenic mice and subsequent Western blot analysis using an NS3-specific antiserum confirming that NS5A does not affect the amount of NS3.

There are two ways by which NS5A could protect NS3/4A-expressing cells from elimination: either to prevent entry of CTLs to the liver or by interfering with one or more of the effector molecule pathways. To investigate whether the entry of CTLs into the liver was affected by NS5A, we monitored hepatic CD3+ cells at the time of elimination by immunohistochemistry. This revealed that comparable amounts of CD3-positive cells were present in wild-type and NS5A-Tg livers. This suggested that NS5A expression did not affect the entry of CTLs into the liver (Fig. 5d). To control the possibility that a reduced effector role of CTLs may cause the impaired elimination of NS3/4A-positive hepatocytes, the lytic activity was determined by a 51Cr-release assay. This revealed a comparable CTL activity in spleens from wild-type and NS5A-Tg mice (Fig. 5e). Taken together, these data indicate that NS5A does not affect entry of T cells into the liver or the activity of CTLs. Thus, this suggests NS5A directly protects the NS3/4A-expressing hepatocytes from elimination by the activated CTLs.

**DISCUSSION**

The role of NS5A on the molecular level has been well characterized, but much less is known about the relevance of NS5A for HCV-associated pathogenesis or its possible role in viral persistence. To gain more insight regarding these issues, we generated a novel transgenic mouse model that displayed a liver-specific expression of NS5A. There is a variety of reports describing the capacity of NS5A to interact with different cellular proteins and thereby modulate intra-
cellular signal transduction cascades (reviewed in Ref. 35). We therefore investigated whether this was reflected by changes in the hepatic gene expression pattern in NS5A-Tg mice. The microarray analysis revealed significant but moderate alterations in 37 genes. At first glance, none of the genes deregulated by NS5A are known to have an obvious association with pathogenic processes. Moreover, promoter analysis of the deregulated genes does not reveal a common pattern of promoter elements shared by NS5A-dependent deregulated genes. Earlier reports on cells stably or transiently producing NS5A of genotype 1b have revealed a variety of genes that are strongly deregulated by NS5A (36). However, comparing these to the list of genes that were significantly deregulated in NS5A-Tg mice, no overlap was identified. This might be due to the difference of the experimental systems and external factors that seem to affect significantly the expression pattern of NS5A-dependent regulated genes. With respect to the moderate deregulation of gene expression in the NS5A-Tg mice, reflecting an equilibrium between transgene-dependent deregulation and compensatory mechanisms, this did not result in a clear phenotype of the NS5A-gt 1a Tg mice, no overlap was identified. This might be due to the difference of the experimental systems and external factors that seem to affect significantly the expression pattern of NS5A-dependent regulated genes. With respect to the moderate deregulation of gene expression in the NS5A-Tg mice, reflecting an equilibrium between transgene-dependent deregulation and compensatory mechanisms, this did not result in a clear phenotype of the NS5A-gt 1a Tg mice (31). However, if this equilibrium between transgene-dependent deregulation and compensatory mechanisms is disturbed, i.e. by a viral infection, the NS5A-transgenics display a clear phenotype. This was demonstrated by impaired clearance of LCMV and reduced induction of 2',5'-OAS or PKR in LCMV-infected NS5A-Tg mice. Apart from its direct role as an essential part of the replication complex (6, 37), NS5A can be considered as an important factor that facilitates the establishment or persistence of the HCV infection. The impaired virus elimination in LCMV-infected mice and the delayed elimination of NS3/4A-positive cells after hydrodynamic injection corroborate this. This suggests that two, possibly independent, mechanisms by which NS5A is able to promote the viral infection are described in this study: impairment of the antiviral interferon response (innate immunity) and the interference with the CTL response (adaptive immunity).

The binding of NS5A genotype 1b to PKR (15) in vivo inhibits PKR that is an essential player for induction and transduction of the interferon-dependent antiviral response (38). In accord with this, the LCMV-infected NS5A-Tg mice had a decreased induction of IFNβ, 2',5'-OAS and PKR, reflecting the inhibitory interference of NS5A with the antiviral interferon response.
Many viruses have the potential to interfere with different steps in the antiviral interferon response to escape the host defense system (39). The HCV NS3/4A complex targets MAVS/IPS-1/VISA/Cardif for cleavage as an immune evasion strategy (40). The NS5A-dependent inactivation of PKR could provide an additional escape mechanism from the host antiviral response and thereby favors viral replication and presumably also persistence. The results from the LCMV infection experiments with the NS5A-Tgs identify NS5A as at least one factor that confers reduced viral clearance in LCMV-infected Tg mice expressing the complete HCV genome (14).

Apart from the effect of NS5A on the innate immune response, the observed NS5A-dependent interference with the CTL response might be an additional factor that confers impaired LCMV elimination in the NS5A-Tgs. The most pronounced differences in LCMV titers were observed after day 6, a time point when T-cell response has been primed. Evidence for the interference of NS5A with the acquired immune response was obtained from the inhibited elimination of NS3/4A-expressing hepatocytes generated by a hydrodynamic injection model. The clearance of NS3-positive cells in this model is exclusively CTL-mediated (24). Because neither CTL activity nor entry of T cells into the liver was altered in the NS5A-Tg mice, NS5A could exert a protective effect on the liver cell. Since the CTL-dependent clearance is in part mediated by TNFα, the inhibitory effect of NS5A on TNFα-dependent apoptosis (41) could be relevant. Moreover, one could speculate that NS5A is the molecule that interferes with Fas-mediated apoptosis through Bid as reported from mice expressing the full HCV polypeptide (42). However incubation of primary hepatocytes derived from wt or NS5A transgenic mice after hydrodynamic injection of pVaxNS3/4A or (right panel) lysate derived from HuH7.5 cells that were cotransfected with a constant amount of pVaxNS3/4A and variable amounts of palbNS5A.

In conclusion, NS5A expression in vivo may help HCV to establish and/or maintain the chronic infection by inhibiting both the innate interferon response and the adaptive CTL response. Considering these new aspects of the relevance of NS5A Impairs Viral Clearance.
NS5A for the HCV infection could help to find new approaches to prevent the establishment of a chronic HCV infection.

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REFERENCES

1. Hoofnagle, J. H. (2002) Hepatology 36, 521–29
2. Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Kato, N., Kimura, K., and Shimotohno, K. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10773–10777
3. Kaneko, T., Tanji, Y., Satoh, S., Hijikata, M., Asabe, S., Kimura, K., and Shimotohno, K. (1994) Biochem. Biophys. Res. Commun. 205, 320–326
4. Penin, F., Dubuisson, J., Rey, F. A., Moradpour, D., and Pawlotsky, J. M. (2004) Hepatology 39, 5–19
5. Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K., and Bartenschlager, R. (2001) J. Virol. 75, 1252–1264
6. Appel, N., Pietschmann, T., and Bartenschlager, R. (2005) J. Virol. 79, 3187–3194
7. Qadri, I., Iwahashi, M., and Simon, F. (2002) Biochem. Biophys. Acta 1592, 193–204
8. Tan, S. L., Nakao, H., He, Y., Vijayrani, S., Neddermann, P., Jacobs, B. L., Mayer, B. J., and Katze, M. G. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 5533–5538
9. Bärcstimmer, T., Kriegs, M., Luberger, J., Pauli, E. K., Schmitt, S., and Hildt, E. (2006) FEBS Lett. 580, 575–580
10. Street, A., Macdonald, A., Crowder, K., and Harris, M. (2004) J. Biol. Chem. 279, 12232–12241
11. Ghosh, A. K., Steele, R., Meyer, K., Ray, R., and Ray, R. B. (1999) J. Gen. Virol. 80, 1179–1183
12. Gale, M. J., Jr., Kwieciszewski, B., Utz, M., Nakao, H., and Katze, M. G. (1999) J. Virol. 73, 6506–6516
13. Leverero, M. (2006) Oncogene 25, 3834–3847
14. Bläckenhagen, A., Duong, F. H., Hunziker, L., Stuven, S. T., Wang, X., Terracciano, L., Moradpour, D., Blum, H. E., Alonzi, T., Tripodi, M., La Monica, N., and Heim, M. H. (2003) Gastroenterology 124, 1465–1475
15. Gale, M. J., Jr., Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polvak, S. J., Gretch, D. R., and Katze, M. G. (1997) Virology 230, 217–227
16. Brinster, R. L., Allen, J. M., Behringer, R. R., Gelinas, R. E., and Palmiter, R. D. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 836 – 840
17. Pinkert, C. A., Ornitz, D. M., Brinster, R. L., and Palmiter, R. D. (1987) Genes Dev. 1, 268–276
18. Hildt, E., Munz, B., Saher, G., Reifenberg, K., and Hofschneider, P. H. (2002) EMBO J. 21, 525–535
19. Chisari, F. V. (1989) Mol. Biol. Med. 6, 143–149
20. Werner, S., Weinberg, W., Liao, X., Peters, K. G., Blessing, M., Yuspa, S. H., Weiner, R. L., and Williams, L. T. (1993) EMBO J. 12, 2635–2643
21. Lauer, U., Weiss, L., Hofschneider, P. H., and Kekulé, A. S. (1992) J. Virol. 66, 5284–5289
22. Bruns, M., Gessner, A., Lother, H., and Lehmann-Grube, F. (1988) Virol- ogy 166, 133–139
23. Saher, G., and Hildt, E. (1999) J. Biol. Chem. 274, 27651–27657
24. Ahlen, G., Nystrom, J., Pult, I., Frelin, L., Hultgren, C., and Sallberg, M. (2005) J. Infect. Dis. 192, 2112–2116
25. Jörns, J., Mangold, U., Neumann, U., Van, Damme, E. J., Peumans, W. J., Pfäffli, U., and Schumacher, U. (2003) Anat. Embryol. 207, 85–94
26. Frelin, L., Ahlen, G., Alheim, M., Weiland, O., Barnfield, C., Liljestrom, P., and Sallberg, M. (2004) Gene Ther. 11, 522–533
27. Frelin, L., Alheim, M., Chen, A., Söderholm, J., Rozell, B., Barnfield, C., Liljestrom, P., and Sallberg, M. (2003) Gene Ther. 10, 686–699
28. Donauer, J., Rumberger, B., Klein, M., Faller, D., Wilpert, J., Sparna, T., Schieren, G., Roehrback, R., Der, P., Timmer, J., Pisarski, P., Kirsie, G., and Walz, G. (2003) Transplantation 76, 539–547
29. Boldrick, J. C., Alizadeh, A. A., Diehn, M., Dudoit, S., Liu, C. L., Belcher, C. E., Botstein, D., Staudt, L. M., Brown, P. O., and Relman, D. A. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 972–977
30. Malingaard, L., Salazar-Mather, T. P., Lewis, C. A., and Biron, C. A. (2002) J. Virol. 76, 4520–4525
31. Majumder, M., Steele, R., Ghosh, A. K., Zhou, X. Y., Thornburg, L., Ray, R., Phillips, N. J., and Ray, R. B. (2003) FEBS Lett. 555, 528–532
32. Assmann-Wischer, U., Simon, M. M., and Lehmann-Grube, F. (1985) Med. Microbiol. Immunol. 174, 249–256
33. Lehmann-Grube, F., Assmann, U., Löglä, C., Moskophidis, D., and Löhler, J. (1985) J. Immunol. 134, 608–615
34. Haller, O., Kochs, G., and Weber, F. (2006) Virology 344, 119–130
35. Macdonald, A., and Harris, M. (2004) J. Gen. Virol. 85, 2485–2502
36. Girard, S., Vossman, E., Misek, D. E., Podevin, P., Hanash, S., Bréchot, C., and Beretta, L. (2004) Hepatology 40, 708–718
37. Penin, F., Brass, V., Appel, N., Ramboarina, S., Montserret, R., Ficheux, D., Blum, H. E., Bartenschlager, R., and Moradpour, D. (2004) J. Biol. Chem. 279, 40835–40843
38. Samuel, C. E. (2001) Clin. Microbiol. Rev. 14, 778–809
39. Hengel, H., Koszinowski, U. H., and Conzelmann, K. G. (2005) Trends Immunol. 26, 396–401
40. Cheng, G., Zhong, J., and Chisari, F. V. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 8499–8504
41. Majumder, M., Ghosh, A. K., Steele, R., Zhou, X. Y., Phillips, N. J., Ray, R., and Ray, R. B. (2002) Virology 294, 94–105
42. Disson, O., Haouzi, D., Desagher, S., Loesch, K., Hahne, M., Kremer, E. J., Jacquet, C., Lemon, S. M., Hibern, U., and Lerat, H. (2004) Gastroenterology 126, 859–872
43. Stobart, M. J., Parchaliuk, D., Simon, S. L., Lemaistre, J., Lazar, J., Rubenstein, R., and Knox, J. D. (2007) Mol. Neurodegener. 2, 5