HEPATITIS

The hepatitis C virus and immune evasion: non-structural 3/4A transgenic mice are resistant to lethal tumour necrosis factor α mediated liver disease

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Background: The hepatitis C virus (HCV) establishes chronic infection by incompletely understood mechanisms. The non-structural (NS) 3/4A protease/helicase has been proposed as a key complex in modulating the infected hepatocyte, although nothing is known about the effects this complex exerts in vivo.

Aim: To generate mice with stable and transient hepatocyte expression of the HCV NS3/4A proteins to study its effects in vivo.

Methods: NS3/4A expression was determined by western blot and immunohistochemistry. Two independent pathologists determined the liver histology. Hepatic immunity was characterised by quantifying intrahepatic immune cell subsets. Liver damage was induced using carbon tetrachloride (CCl₄), lipopolysaccharide (LPS), tumour necrosis factor α (TNF α), and anti-Fas antibody.

Results: Expression of NS3/4A was restricted to the cytoplasm of hepatocytes, and did not cause liver cancer or any spontaneous liver pathology. However, the presence of NS3/4A modulated the intrahepatic immunity, as follows: first, the CD4⁺ T cell and type I/II dendritic cell subsets were reduced in transgenic livers; second, NS3/4A protected hepatocytes from liver damage mediated in vivo by CCl₄, LPS, TNF α , but not FAS; and third, both stable and transiently NS3/4A transgenic mice were resistant to lethal doses of liver targeted TNF α , and the resistance could be reverted by treatment with a p38 mitogen activated protein kinase inhibitor (MAPK).

Conclusions: Hepatic expression of NS3/4A does not induce spontaneous liver disease. NS3/4A does, however, alter the intrahepatic immune cell subsets and protects hepatocytes against TNF α induced liver damage in vivo. The TNF α resistance can be reverted by treatment with a p38 MAPK inhibitor. This represents a new immune evasion strategy conferred by NS3/4A.

The hepatitis C virus (HCV) is a major cause of chronic liver disease, affecting more than 170 million people worldwide.¹ Most HCV infections become chronic, for reasons not yet fully understood. The slow development of the disease and the rather weak immune responses suggest that HCV infection itself has a limited immunogenicity.² The HCV 9.4 kb linear, single stranded, positive sense RNA genome encodes a polyprotein that is processed to 10 structural and non-structural (NS) proteins.³ Using the replicon system it has been shown that HCV replication is sensitive to interferons (IFN) α/β and γ , but resistant to tumour necrosis factor α (TNF α) and double stranded (ds) RNA.^{4–6}

It has been suggested that HCV evades the host immune response by several mechanisms. First, the HCV genome has a high plasticity which allows immune evasion by mutations within epitopes recognised by the host immune response.⁷ However, the ability to undergo mutational immune escape may be restricted by effects on the viral fitness.⁸ Thus HCV also uses several other mechanisms to escape the host response. First, HCV core and envelope proteins seems to mediate resistance to cytokines including TNF α .⁹ Second, the resistance of HCV replicons to IFN and dsRNA has been explained in vitro by the fact that the NS3/4A proteins block the cellular IFN response by any or all of the following:

- inhibition of IFN regulatory factor (IRF)-3 phosphorylation¹⁰;

- disruption of retinoic acid inducible gene-I (RIG-I) signalling¹¹ by cleavage of the caspase recruitment domain adaptor inducing IFN β (CARDIF,¹² also known as IPS-1¹³ or MAVS¹⁴);
- inactivation of TOLL-like receptor (TLR) 3 signalling by proteolytic cleavage of the adaptor molecule Toll/IL-1 receptor domain containing adaptor inducing IFN β (TRIF)¹⁵;
- binding to TANK binding kinase-1 (TBK1).¹⁶

However, any effect of NS3/4A expression in vivo is not yet known.

As HCV does not infect mice, different transgenic (Tg) mice expressing the HCV core,^{17–18} core and envelope (E) 1 and E2,¹⁹ NS5A,²⁰ and the full length (fl) HCV polyprotein^{19–21} have been generated. Many of these—in particular those expressing the HCV core protein—develop spontaneous liver disease and hepatocellular carcinoma (HCC),^{17–19} and those expressing the NS proteins have an impaired clearance of hepatotropic viral infections.^{19–22} Finally, in humans and flHCV-Tg mice, increased levels of the protein phosphatase

Abbreviations: D-Gal, D-glucosamine; HCV, hepatitis C virus; IP-WB, immunoprecipitation and western blot; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase inhibitor; NS, non-structural; RPA, RNase protection assay; TBK1, TANK binding kinase-1; Tg, transgenic; TNF α , tumour necrosis factor α ; TRIF, TIR domain containing, adapter inducing interferon β

(PP) 2A caused impaired activation of signal transducer and activator of transcription (STAT) 1,²² which may help to explain the impaired immunity. It was recently suggested that this was caused by the helicase activity of NS3.²³ However, the NS protein that modulates the host immune response in vivo is not yet known. As one or more HCV proteins blocks intrahepatic immunity to promote viral persistence we generated a transgenic mouse to explore the role of the NS3/4A complex.

METHODS

Generation of stable NS3/4A transgenic mice

A full length wild type HCV genotype 1a NS3/NS4A gene with a functional protease (amino acids 1026 to 1711)^{8, 24} was fused to the mouse major urinary protein (MUP) promoter using BamHI and EcoRI sites of pMUP-11AS plasmid (kindly provided by Dr William Held, Roswell Park Cancer Institute, Buffalo, New York, USA).²⁵ The entire pMUP-NS3/4A-BGH polyadenylation signal construct was sequenced before

Figure 1 Characterisation of transgenic mice with hepatic expression of NS3/4A. Schematic representation of the linearised MUP-NS3/4A fragment used for microinjection to produce the stable transgenic mice (A), and the pVAX1 plasmid expressing the codon optimised NS3/4A gene²⁸ used to generate the transiently transgenic mice (A). Also shown is the detection of the full length integrated NS3/4A transgene by polymerase chain reaction (PCR) in individual stable NS3/4A-Tg mice (B; lanes 3 to 6) and a non-Tg control mouse (B; lane 7). Lanes 1 and 2 show the molecular weight markers Lambda DNA, Hind III Digest, and ϕ X-174-RF DNA, Hae III Digest (Amersham Pharmacia Biotech, Uppsala, Sweden). Lane 8 shows a negative PCR control and lane 9 a positive PCR control (wtNS3/4A plasmid DNA). Immunoprecipitation and western blot analysis of HCV NS3 protein expression in liver homogenates from stable NS3/4A-Tg mice and a non-Tg littermate (C). NS3 proteins were found in the stable NS3/4A-Tg mice (C; lanes 4 to 6), and neither protein was found in a non-transgenic littermate (C; lane 2). HepG2 cells transiently transfected by a codon optimised NS3/4A gene (C; lane 3) served as a positive control. Also shown is the molecular weight marker in lane 1 (Magic Mark, Invitrogen). In (D) it is shown that the band identified in the transient (lane 4) and stable (lane 5) NS3/4A-Tg mice corresponds to cleaved NS3, as the band size is the same as the only band seen in BHK cells expressing NS3 alone (lane 6), and the lower molecular weight band in NS3/4A expressing BHK cells (lanes 2, 3, and 8). The band is also smaller than the uncleaved NS3-NS4A band seen in BHK cells transfected with a mutant NS3/4A protein with a defective proteolytic site between NS3 and NS4A (lane 7²⁴). Lane 1 shows the molecular weight marker, lane 9, mock transfected BHK cells, and lane 10, a non-Tg mouse liver. Histological analysis of livers from stable NS3/4A-Tg mice (E, F, and J) and non-Tg littermates (E, F, and G). Liver sections were stained with haematoxylin and eosin (E, F, H, and I) or with oil red O (G and J) according to standard procedures. Liver sections were obtained from two month old (E and H) or >17 month old (F, G, I, and J) stable transgenic and non-transgenic littermates. Photographs were obtained with an original magnification of $\times 40$ (E and H) and $\times 100$ (F, G, I, and J).

microinjection to confirm the correct sequence. The construct was microinjected into fertilised oocytes from C57BL/6 $\times$ CBA F1 (F1) mice, using standard techniques, at the Unit for Embryology and Genetics, Karolinska Institutet, Sweden. Founder mice were analysed for the presence of genomic transgene DNA by polymerase chain reaction (PCR). For detection of NS3/NS4A by PCR, we used specific primers—that is, sense primer: 5'-CCT GAA TTC ATG GCG CCT ATC ACG GCC TAT-3'; antisense primer: 5'-CCG TCT AGA TCA GCA CTC TTC CAT TTC ATC-3'.

Generation of transiently NS3/4A transgenic mice

Transiently transgenic mice were generated as previously described.^{26, 27} In brief, groups of five to 10 naive wild type F1 18–20 g mice were injected intravenously in the tail vein in less than 10 seconds with 1.8 ml Ringer's solution containing either an empty pVAX1 plasmid (Invitrogen, San Diego, California, USA), or a pVAX1 plasmid containing a codon-optimised NS3/4A (coNS3/4A) gene.²⁸ All experiments were done 120 hours after the hydrodynamic injection to allow liver function a sufficient time to normalise. All mice used were housed in microfiltered cages in a controlled and pathogen-free environment.

Detection of NS3 by immunoprecipitation and western blot

Detection of NS3 by immunoprecipitation and western blot (IP-WB) was carried out essentially as described.²⁸ In brief,

organ samples (100 mg) were homogenised and lysed in 1 ml radioimmune precipitation assay (RIPA) buffer (0.15 M NaCl containing 50 mM Tris, 1% Triton-X 100, 1% sodium deoxycholate, and 1% sodium dodecyl sulphate (SDS)). The homogenates were immunoprecipitated with protein A sepharose, loaded with polyclonal anti-NS3 antibody overnight at 4°C. The washed pellets were re-suspended, heated at 100°C for five minutes, and then loaded on a 4–12% Bis-Tris gel (Invitrogen) for SDS-PAGE analysis. Proteins were then transferred to nitrocellulose membranes and NS3 protein was detected by chemiluminescence according to manufacturer's protocol (WesternBreeze; Invitrogen). Chemiluminescent signals were detected using the GeneGnome equipment (Syngene, Cambridge, UK). Expression levels were quantified using a standard curve of rNS3.

Biochemical analysis

Serum (s) samples were tested for alanine transferase (sALT; Modular P apparatus; Roche Diagnostics, Mannheim, Germany) and blood glucose levels by using the Accu-Check® Sensor equipment (Roche Diagnostics).

Histology and immunohistochemistry

Mice were killed and liver tissue was placed in formalin or OCT (Tissue-Tek, Torrance, California, USA) and immediately snap frozen in liquid nitrogen. Formalin fixed liver samples were paraffin embedded and sectioned. Liver sections 4 μ m

Figure 2 Characterisation of transgene expression in NS3/4A-Tg mice with an integrated transgene (stable Tg; A to D), or with a transient transgene (transient Tg; E and F). Hepatic HCV NS3 protein expression was detected by immunoprecipitation and western blot (IP-WB) (A and E) and by immunohistochemistry (B to D and F). In the mice with an integrated NS3/4A transgene, NS3/4A protein expression was liver (Li) specific (A; lane 4), and was not detected in spleen (Sp; A, lane 5) or kidney (Ki; A, lane 6), or in non-Tg littermates (A; lanes 1 to 3). Lane 7 shows a recombinant NS3 helicase domain protein, "M" (both A and E) molecular weight markers (Magic Mark, Invitrogen), and "+" (both A and E) transiently NS3/4A transfected HepG2 cells. Liver sections were stained with a human anti-NS3 antisera (B to D and F). Sections were photographed at an original magnification of $\times 400$ (B), $\times 200$ (C and D), and $\times 40$ (F). In (E), detection of NS3/4A expression by IP-WB in livers from three separate mice per gel at 96 and 120 hours after hydrodynamic injection of the codon-optimised NS3/4A plasmid (transient Tg) has been given. The three mice with integrated NS3/4A (stable Tg) are the same in both gels. In (F) the section was obtained 48 hours after hydrodynamic injection.

thick were mounted on slides and stained with haematoxylin-eosin dye. Liver sections were also stained with periodic acid Schiff (PAS) for detection of basement membranes and glycogen accumulation, and Sirius red staining for visualisation of fibrosis. Frozen liver sections (4 to 7 μ m thick) were stained using oil red O to visualise lipid droplets and estimate the degree of macro- and microsteatosis. The different stainings were done following standard procedures. Sections were analysed by two independent pathologists blinded to the transgene status of the animals.

Cellular apoptosis or necrosis was measured by detecting DNA strand breakage using the TUNEL (TdT mediated dUTP-biotin 3'-OH nick-end labelling) assay according to the manufacturers protocol (ApoTag peroxidase in situ apoptosis detection kit, Chemicon, Germany).

NS3 was detected in microwave pretreated, paraffin embedded sections using a human anti-NS3 antibody and biotinylated anti-human IgG (Vector Laboratories, Burlingame, California, USA).

Isolation of intrahepatic immune cells and flow cytometry

Male NS3/4A-Tg or non-Tg mice (6–12 weeks old) were killed. Livers were perfused with 5–7 ml of phosphate buffered saline (PBS; pH 7.4, 20°C) and then excised and weighed. In each experiment, 100–125 mg of liver tissue were taken for western blot analysis or immunohistochemistry, or

both. The remaining liver tissue was placed in 5 ml of serum-free AIM-V medium (Gibco-BRL). A single cell suspension was prepared by crushing the organ in culture medium, passed through a 70 μ m cell strainer, and then centrifuged for 15 minutes at 200 $\times$ g. The cell pellet was resuspended in 80% isotonic Percoll solution (Amerham Pharmacia Biotech) and overlaid with a 40% Percoll solution. The cells were then centrifuged for 20 minutes at 300 $\times$ g and the interphase was collected for flow cytometry analysis as described below.

The isolated cells were incubated for 20 minutes with Fc block (anti-CD16/CD32 clone 2.4G2), and then on ice for 30 minutes with fluorochrome or biotin conjugated monoclonal antibodies (mAbs). After washing, cells stained with biotinylated antibodies were exposed to streptavidin conjugated phycoerythrin (PE) for 30 minutes on ice. The following antibodies (all from BD Biosciences, San José, California, USA) were used: anti-CD16/CD32 clone 2.4G2; FITC conjugated anti-CD3 (145-2C11), anti-CD11c (HL3); PE conjugated anti-NK1.1 (PK136); biotin conjugated anti-MHC class I-A/I-E (2G9), anti-CD4 (RM4-5); PerCP-Cy5.5 conjugated anti-CD11b (M1/70), anti-CD19 (1D3); and APC conjugated anti-CD45 (Ly-5, 30-F11), anti-Ly 6C/G (RB6-8C5), anti-CD8 (53-6.7). Appropriate isotype controls (all from BD Biosciences) were used to check for background staining and for setting gates. A minimum of 50 000 propidium iodine negative (that is, live) leucocytes (CD45 positive cells backgated on forward/side scatter profile) was

Figure 3 Analysis of intrahepatic immune cell populations in stable NS3/4A-Tg and non-Tg mice by flow cytometry. In (A) the detected marker and the probable cellular subset has been indicated. Also shown is the analysis of intrahepatic dendritic cell (DC) populations in stable NS3/4A-Tg and non-Tg mice by flow cytometry (B and C). In (B) the definition of the different DC subsets according to expression of MHC class II and Ly6C/G by staining with antibodies against CD11b, MHC II, and Ly6C/G has been shown. In (C) the mean percentage of the three different DC subsets in groups of 10 stable NS3/4A-Tg and non-Tg mice is shown. * $p < 0.05$; *** $p < 0.001$ (Student's t test).

Figure 4 Stable NS3/4A-Tg mice are resistant to TNF α mediated liver damage. Liver damage was determined by serum alanine transaminase (ALT) levels after injection of CCl₄ (A), LPS/D-Gal (B), TNF α /D-Gal (C); only ALT levels from surviving non-Tg mice, and FAS antibody Jo2 (D) in non-Tg and stable NS3/4A-Tg mice. The mice were monitored for 48 to 72 hours. Error bars = SD. D-Gal, D-glucosamine; LPS, lipopolysaccharide; TNF α , tumour necrosis factor α .

analysed per sample. FACS data were acquired on a FACSCalibur (BD Biosciences) and data analysed with CellQuest software (BD Biosciences).

Rnase protection assay (RPA)

Total RNA was extracted from NS3/4A-Tg and non-Tg mice liver homogenates using TRIzol Reagent (Gibco-BRL) according to the manufacturer's protocol. In brief, total RNA was extracted and purified (RNeasy, Qiagen, Valencia, California, USA). All RNA samples used in the RPA had an A_{260/280} ratio of >1.9. Twenty micrograms of total RNA were assayed with the Multi-probe RPA system using the probe template set mCD-1 according to the manufacturers recommendations (BD Biosciences). In brief, RNA was hybridised overnight with [α -³²P]dUTP labelled mCD-1 (7 $\times$ 10⁵ cpm) and digested with RNase A and T1. Rnase protected probes were purified by phenol extraction and resolved on a 4.75% denaturing polyacrylamide gel together with the non-protected probe and subjected to autoradiography. Using the undigested probes as markers, a standard curve of migration distance versus nucleotide lengths was plotted. This standard curve was used to identify the "Rnase protected" fragment lengths.

Induction of liver damage

Liver injury was monitored using sALT levels or survival in groups (n = 5–10) of 6–12 week old mice. Carbon tetrachloride (CCl₄) 0.5 g/kg in 100 μ l olive oil was given intraperitoneally (ip). Lipopolysaccharide (LPS; 5 μ g/kg)/D-glucosamine (D-Gal; 20 mg; Sigma) was given ip in 100 μ l PBS. D-Gal specifically blocks transcription in hepatocytes, which results in an in vivo model where the mice are sensitised to liver damage caused by TNF α or LPS.²⁹ Anti-mouse Fas monoclonal antibody (0.1 mg/kg; Jo2; BD Biosciences) was given in 200 μ l PBS intravenously (iv). TNF α at doses of

5–20 μ g/kg with D-Gal (20 mg) was given ip in 100 μ l PBS containing 1 mg/ml bovine serum albumin (Sigma). To block the p38 MAPK, 25 mg/kg SB203580 (Calbiochem) in sterile dH₂O was given intraorally 30 minutes before TNF α /D-Gal injections.

Ethics

All experimental protocols involving animals were approved by the local ethics committee for animal experimentation.

RESULTS

Characterisation of transgenic mice with stable and transient NS3/4A expression

The two different DNA constructs used to generate the stable and transient transgenic mice have been schematically described in fig 1A. NS3/4A DNA transgene positive mice were identified by PCR (fig 1B). One of six DNA positive lineages had hepatic expression of the NS3/4A complex (fig 1C, lanes 4 to 6). We were able to detect NS4A by IP-WB in transiently transfected BHK cells, but not in Tg livers (data not shown). However, as NS4A is expressed as a fusion protein with NS3 which is post-translationally cleaved into NS3 and NS4A, the inability to detect NS4A in livers most probably reflects technical limitations. Importantly, we could confirm that the in vivo expressed NS3 encompassed an active protease domain, as the protein band in the NS3/4A-Tg livers (fig 1D, lanes 4 and 5) corresponded to the full length NS3 protein (fig 1D, lane 6). Also, the band was smaller than the NS3-NS4A fusion band seen in BHK cells expressing an NS3/4A protein with a defective protease site between NS3 and NS4A (fig 1D, lane 7). The identity of two lower molecular weight bands corresponding to sizes of 30–40 kDa which are detectable by IP-WB in mice and cells with NS3/4A

Figure 5 Survival after administration of $\text{TNF}\alpha$ /D-Gal was monitored for 120 hours in stable NS3/4A-Tg mice (A) and transiently NS3/4A-Tg mice (B). Transiently transgenic mice were generated by hydrodynamic injection of 100 μg of codon-optimised NS3/4A plasmid (NS3/4A; $n = 10$) or empty pVAX1 (pVAX1; $n = 11$) plasmid in wild type F1 mice, or untreated wild type F1 mice ($n = 10$), and all were given the same $\text{TNF}\alpha$ /D-Gal dose. Values in (A) and (B) are given as the percentage of surviving mice and were compared using Fisher's exact test. Also shown is the histological appearance in haematoxylin-eosin (HE) (C) or TUNEL (C) stained liver sections from non-Tg or stable NS3/4A-Tg mice at 240 and 480 minutes after $\text{TNF}\alpha$ /D-Gal treatment. In the TUNEL (C) stained liver sections, nuclei with evidence of DNA fragmentation are brown. Frequencies were compared using Fisher's exact test and probability (p) values are indicated. A p value >0.05 was considered non-significant (NS). D-Gal, D-glucosamine; LPS, lipopolysaccharide; $\text{TNF}\alpha$, tumour necrosis factor α ; TUNEL, TdT mediated dUTP-biotin 3'-OH nick-end labelling.

expression (fig 1C and 1D) has not yet been determined, but may represent additional cleavage of NS3 and/or NS4A.

Alanine transaminase (ALT), glucose levels, and liver histology (data not shown, and fig 1E, 1F, 1H, and 1I) did not differ between Tg and non-Tg mice. Additional staining showed no difference in glycogen storage (data not shown), or macro- or microvesicular steatosis (fig 1G and 1J), between Tg and non-Tg livers. No signs of fibrosis or cirrhosis were present for up to >17 months in age matched Tg and non-Tg mice (data not shown). HCC was not more common in the NS3/4A-Tg mice as HCC was detected in one of 183 Tg mice and in none of 148 non-Tg mice. Thus intrahepatic expression of NS3/4A did not induce any detectable liver pathology.

Expression patterns of NS3/4A in stable and transient transgenic mice

As expected when using the MUP promoter a range of NS3/4A expression levels were detected, ranging from 0.1 to 8 μg NS3/4A per g liver tissue (fig 2A and data not shown). The expression levels in the stable NS3/4A-Tg mice were determined by correlating the respective NS3 band densities with the densities of a serial dilution of recombinant NS3 analysed by IP-WB (data not shown). Livers from homozygote stable transgenic mice were positive for NS3 by both immunohistochemistry and IP-WB, or by IP-WB alone (figs 1 and 2, and data not shown). Thus immunohistochemistry is unable to visualize the total number of NS3 expressing cells correctly, but can estimate the number of high level NS3

expressing cells and such cells constituted 1–5% of stable Tg hepatocytes (fig 2B to 2D). The subcellular distribution of NS3/4A was strictly cytoplasmic, which is fully consistent with the appearance of NS3 in infected humans³⁰ (fig 2B to 2D).

In the transiently transgenic mice, both expression levels (fig 2E) and the subcellular localisation (fig 2F) of NS3 were the same as in the integrated NS3/4A-Tg mouse livers. We were able to detect transient intrahepatic expression of NS3/4A for more than 120 hours (fig 2E), and the number of hepatocytes with high level NS3/4A expression as determined by histology ranged from 5% to 20% of hepatocytes in different experiments (fig 2F, and data not shown). Thus these two independent transgenic models show expression of NS3/4A at comparable levels and with an identical subcellular distribution.

NS3/4A-Tg livers have altered hepatic immune cell subsets

An analysis of the intrahepatic immune cell populations revealed that stable NS3/4A-Tg livers showed a marked reduction in CD4⁺ T cells as determined both by flow cytometry (fig 3A) and by mRNA expression using the RPA (data not shown). Moreover, we also found that the NS3/4A-Tg livers had alterations in the dendritic cell subsets (fig 3B and 3C). In particular, we found that the CD11c⁺/CD11b⁻/MHC-II int/Ly6C⁺/G⁺ population (termed type I/II dendritic cells)—which has a similar antiviral phenotype as the type-I IFN producing plasmacytoid dendritic cells^{31–32}—was

Figure 6 The resistance to TNF α mediated liver disease can be reverted by a p38 MAPK-inhibitor. Basal levels of phosphorylated (p) p38 MAPK in stable NS3/4A-Tg and non-Tg mice as determined by western blot (A). Also shown is the change in p-p38 MAPK levels after TNF α /D-Gal treatment with or without the p38 MAPK inhibitor SB203580 (25 mg/kg). The effects on survival of blocking p38 MAPK in vivo during TNF α /D-Gal induced liver damage were tested in groups of 10 stable NS3/4A-Tg (C) or non-Tg mice (D) given 0.3 μ g TNF α /D-Gal with or without SB203580 (25 mg/kg), and survival was monitored for 120 hours. Differences were compared at 120 hours using the Fisher's exact test, and a p value of >0.05 was considered non-significant (NS). D-Gal, D-glucosamine; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase inhibitor; TNF α , tumour necrosis factor α .

significantly reduced in the NS3/4A-Tg livers (fig 3C). Taken together, this suggested that the hepatic expression of NS3/4A might directly or indirectly affect the hepatic immunity.

NS3/4A protects the liver from lethal TNF α mediated liver disease

To address any changes in functional immunity in the transgenic livers we treated mice with substances that induce liver damage by different mechanisms. These treatment studies showed that the stable NS3/4A-Tg mice had a reduced sensitivity to liver damage induced by CCl₄, LPS/D-Gal, and TNF α /D-Gal, as determined by serum ALT (fig 4A to 4C). All these compounds cause a liver disease involving TNF α . First, CCl₄ (fig 4A) initially causes toxic liver injury but the subsequent inflammation is TNF α dependent.³³ LPS-TLR4 activation (fig 4B) causes liver damage by TNF α production from macrophages/Kupfer cells.³⁴ Finally, soluble TNF α (fig 4C), which activates the hepatocyte TNF receptor-1 (TNFR1), induces apoptotic signalling.³⁵ The resistance to induced liver damage was limited to TNF α as the anti-Fas antibody Jo2 caused comparable liver damage in Tg and non-Tg mice (fig 4D).

We also carried out experiments to monitor the survival after treatment with liver targeted TNF α . Injection of TNF α /D-Gal caused a liver disease killing 80% of the non-Tg mice, whereas all stable NS3/4A-Tg mice survived the same treatment (fig 5A; $p < 0.05$, Fisher's exact test). Importantly, transiently NS3/4A-Tg mice generated by a hydrodynamic injection²⁶ were as resistant to TNF α as the mice with an integrated transgene (fig 5B). Although around 5–20% of the hepatocytes of the transiently Tg livers express high levels of NS3/4A (most probably underestimated because of the poor

sensitivity of immunohistochemistry), the survival of these cells is clearly enough to ensure survival of the mouse. Thus the NS3/4A mediated resistance to TNF α mediated liver damage is dependent on the presence of NS3/4A and can be reproduced in two completely independent transgenic models.

Histological analysis of the stable NS3/4A-Tg, and corresponding non-Tg animals, after TNF α /D-Gal treatment showed that only limited histological changes—including vesiculation of hepatocyte nuclei—were seen up to 240 minutes post-treatment. However, an increased number of TUNEL positive hepatocyte nuclei was seen in the non-Tg animals, whereas only scattered positive nuclei were observed in the stable NS3/4A-Tg animals determined by TUNEL staining (fig 5C, and data not shown). At 480 minutes post-treatment, both the stable NS3/4A-Tg and non-Tg mice showed very extensive and severe changes in the liver parenchyma. Extensive areas of haemorrhage were predominately localised around the central veins (fig 5C). In addition, massive numbers of dying/dead cells showing features of apoptosis were evident. In contrast, the areas surrounding the portal triads were relatively spared. TUNEL staining showed a zonal distribution of positive cells, mainly in the areas surrounding the central veins, although scattered positive hepatocyte nuclei were also seen elsewhere (fig 5C). In the non-Tg livers positive cells had a more swollen appearance, suggesting more extensive hepatocyte necrosis compared with the stable NS3/4A-Tg animals (fig 5C). Thus TNF α /D-Gal induces lethal liver failure in non-Tg mice but fails to do so in the NS3/4A-Tg mice, suggesting that NS3/4A interferes with one or more steps involved in TNF α mediated apoptosis or necrosis.

Figure 7 A summary cartoon of previously reported and presently described (for $\text{TNF}\alpha$) inhibition of intracellular signalling pathways identified in vivo (Fas⁴⁰ and IFN^{22}) and in vitro (IFN ,⁴¹ TLR3 ,¹⁵ and RIG-I). IFN , interferon; RIG-I , retinoic acid inducible gene I; $\text{TNF}\alpha$, tumour necrosis factor α ; TLR3 , Toll-like receptor 3.

Resistance to $\text{TNF}\alpha$ can be reverted by p38 MAPK inhibitors

A new class of pharmaceuticals, termed p38 MAPK inhibitors, has been tested in clinical trials with the aim of reducing the effects of $\text{TNF}\alpha$.³⁶ The specificity of these inhibitors is not yet fully understood, suggesting that they may block several pathways. Many lines of evidence are emerging that implicate MAPK signalling, in addition to the classical nuclear factor κB (NF κB) pathway, in the regulation of $\text{TNF}\alpha$ mediated apoptosis.^{37–39} We therefore tested whether a p38 MAPK inhibitor which is well tolerated in vivo could affect the resistance to $\text{TNF}\alpha$ in the stable NS3/4A-Tg mice.

We first analysed the basal expression levels of the phosphorylated p38 MAPK in non-Tg and stable NS3/4A-Tg liver homogenates and found that these were comparable (fig 6A). We next tested if co-administration of the SB203580 p38 MAPK inhibitor and $\text{TNF}\alpha/\text{D-Gal}$ resulted in an inhibition of p38 MAPK activation, which it did (fig 6B). We could now test the effects of the p38 MAPK inhibitor on survival, and we co-administered $\text{TNF}\alpha/\text{D-Gal}$ and the p38 MAPK inhibitor SB203580 to stable NS3/4A-Tg and non-Tg mice. Consistent with our previous observations, the NS3/4A-Tg mice were resistant to lethal doses of $\text{TNF}\alpha/\text{D-Gal}$ whereas most non-Tg mice died (fig 6C and 6D). Co-administration of SB203580 completely reverted the $\text{TNF}\alpha$ resistant phenotype of the stable NS3/4A-Tg mice to a wild type level of sensitivity (fig 6C), but had no effect on $\text{TNF}\alpha$ sensitivity in non-Tg mice (fig 6D). Thus the HCV NS3/4A mediated resistance to $\text{TNF}\alpha$ is reversible by a p38 MAPK inhibitor, which suggests new directions for HCV treatment. This also reveals a previously unknown way by which the NS3/4A complex interferes with the antiviral response of the host cell and immune response (fig 7).

DISCUSSION

There is little doubt that the NS3/4A complex exerts multiple effects on the infected hepatocyte and the surrounding immune cells. These effects are most probably of key importance as the ability of NS3/4A to undergo extensive immune escape mutations is limited by the viral fitness.⁸ We here describe a new immune evasion strategy conveyed by HCV NS3/4A in vivo which renders the hepatocyte resistant to $\text{TNF}\alpha$ mediated apoptosis, a key molecule in cytotoxic T cell mediated elimination of virus infected hepatocytes.⁴²

To study the effects of HCV NS3/4A in vivo we generate new stable and transiently transgenic mice. Using these mice we found, unlike what has been found in other HCV protein

transgenic models,^{17–19} no evidence that hepatic NS3/4A-expression causes any spontaneous liver disease. However, we did find that the NS3/4A protein exerts some important effects on the liver, such as altering intrahepatic immune cell subsets and conferring resistance to lethal $\text{TNF}\alpha$ mediated liver damage. We do not yet know if, or how, these two effects may be connected. For example, we could recently show that a HCV NS3/4A based genetic vaccination of transiently transgenic mice resulted in entry, or a constant circulation as previously suggested,⁴³ into the liver of specific cytotoxic T lymphocytes that eliminated most NS3/4A expressing hepatocytes within 72 hours.²⁶ If liver homing of T cells and killing of hepatocytes is negatively affected by hepatic NS3/4A, this clearance may have occurred more rapidly, which needs further study. This new immune escape mechanism conferred by NS3/4A may help to explain why HCV replication is resistant to $\text{TNF}\alpha$,^{4–5} and how high serum levels of $\text{TNF}\alpha$ as well as $\text{TNF}\alpha$ producing intrahepatic CTLs, can co-exist with a high viral replication.⁶

A key observation was that the HCV NS3/4A induced resistance to $\text{TNF}\alpha$ could be reverted in vivo by treatment with a specific p38 MAPK inhibitor. This has several implications. First, it suggests that p38 MAPK inhibitors might have a place in the treatment of chronic HCV infections to counteract the effects of NS3/4A, which certainly needs to be explored further. Second, the dependence on pathways which are blocked by the p38 MAPK inhibitor suggests that these pathways could be affected by NS3/4A. For example, p38 MAPK has been suggested to inhibit pro-apoptotic pathways by activation of PP2A.³⁹ This would be consistent with the previously reported increased levels of PP2A in livers from humans and mice expressing the full length HCV polyprotein.²² However, other reports suggest that inhibition of PP2A may inhibit $\text{TNF}\alpha$ mediated apoptosis.³⁸ Thus the role of NS3/4A and its interaction with pathways affected by the p38 MAPK inhibitor in apoptotic signalling needs to be fully understood.

In conclusion, HCV NS3/4A interferes with multiple steps involved in intracellular responses against viral pathogens, such as the blocking of IRF-3 phosphorylation,^{10–11} inactivation of TRIF,¹⁵ or binding to TBK1.¹⁶ Together with the results reported herein, this rather remarkable range of anti-inflammatory mechanisms mediated by the NS3/4A complex may be necessary to confer HCV persistence, given the high level of redundancy in innate and adaptive antiviral immune responses. $\text{TNF}\alpha$ resistance should favour chronicity and represents a new evasion strategy mediated by NS3/4A in vivo. In some models virally infected hepatocytes have been reported to be intrinsically resistant to perforin mediated killing,⁴⁴ which leaves the death receptors Fas and TNFR1/2 as a means of immunological eradication of HCV infected cells. However, published and preliminary observations suggest that both CTLs²⁶ and perforin participate in the clearance of HCV NS3/4A expressing hepatocytes (Ahlén G *et al*, unpublished observations). As it has been proposed that HCV interferes with Fas mediated killing and we show here that HCV also impairs the $\text{TNF}\alpha$ pro-apoptotic signalling pathway, this may well help to explain why HCV so effectively establishes chronic infections. The exact mechanism by which NS3/4A exerts this effect needs to be defined. These mice will also be instrumental for in vivo studies on the previously reported in vitro effects that the NS3/4A complex exerts on different signalling pathways. As a final speculation, one could suggest that the use of p38 MAPK inhibitors in the treatment of HCV merits further investigation.

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