Supplemental online data:
Methods:

Microarray

Complementary DNA (cDNA) microarrays were produced and processed essentially according to the Stanford protocol described by Eisen and Brown. A list of all the 15,000 genes on the chip is available on the home page of the Core Facility Genomics Web site (www.genomics.uni-freiburg/products/genelist). Sample or reference RNA was transcribed into cDNA in the presence of Cy3- or Cy5-labeled dUTP, respectively. Hybridizations were performed in the presence of an equal amount of reference RNA (Stratagene, La Jolla, CA) as described by Boldrick et al. (29). All other steps, including hybridization, were performed following the protocol published by Brown et al. (http://cmgm.stanford.edu/pbrown).

^{51}Cr-release assay

In brief, mice were immunized with coNS3/4A-pVAX1 or left untreated. 13 days post immunization, a hydrodynamic injection of coNS3/4A-pVAX1 was performed. Three days after the hydrodynamic injection, spleenic CTLs were isolated. CTLs were detected using an NS3-derived H-2D^{b}-restircted CTL peptide. Peptide loaded RMA-S cells incubated with ^{51}Chromium (GE Healthcare) were used as target cells. Specific lysis was calculated as followed:

lysis [%] = ((sample – minimum release) / (maximum release – minimum release)) x 100

specific lysis [%] = sample with peptide loaded target cells – sample without peptide l. t. c.

Isolation and cultivation of primary mouse hepatocytes

Primary mouse hepatocytes were isolated from NS5A transgenic and wt mice with a two-step collagenase perfusion as previously described (Klingmuller, Bauer et al. 2006). Cells were plated on collagen-coated tissue culture dishes in William's medium E (WME) supplemented with 10% FCS, 100 nM dexamethasone, 2 mM l-glutamine and 1%-penicillin/streptomycin solution. For induction of apoptosis cells were incubated with 20ng/ml TNFα for 24h.

Results

Increased phosphorylation of Raf-1 in NS5A transgenic mice (supplement)

In vitro experiments have demonstrated that NS5A binds to and activates Raf-1 (9). The NS5A-dependent activation of Raf-1, however, does not result in an activation of MEK/MAP2 kinase. Co-immunoprecipitation experiments of liver-derived lysates showed the capacity of NS5A to bind to Raf-1 in vivo (suppl. fig. 1a). To investigate whether NS5A also affects Raf-1 phosphorylation in vivo, western blotting using a phospho-Raf-specific antiserum (Ser338) was performed. The western blot shows an increased amount of phosphorylated Raf-1 in lysates derived from the NS5A-transgenic mice as compared to the corresponding wildtype control (suppl. fig. 1b). Lysates derived from mice producing the HBV regulatory protein MHBs^{t76}, a known activator of the Raf-1/MEK/MAP2 kinase (18), served as positive control. Analyzing Raf-1 activity by immunocomplex assay indicates that the observed increased phosphorylation of Raf-1 in NS5A-transgenic mice is associated with an increased activation (suppl. fig. 1c). Probing of the membrane with anti-active MEK (suppl. fig. 1d) or anti-active MAP2 kinase-specific antisera (suppl. fig. 1e) revealed no difference in the amount of pMEK or pMAP2 kinase between wildtype and NS5A-transgenic animals. In case of the MHBs^{t76} transgenic mice, increased amounts of pMEK and pMAP2 kinase were detectable. This indicates that NS5A activates Raf-1 in vivo and that this activation triggers downstream events distinct of MEK/MAP2 kinase activation.

Activation of Raf-1 can induce an increased proliferation rate. The proliferation status of the liver cells was determined by analysis of the PCNA level. Quantification of the PCNA level by western blot analysis revealed that there is no significant difference between wildtype and NS5A-transgenic animals (suppl. fig. 1f). In agreement with that, the size and the mass of livers derived from transgenic mice and wild type mice are similar (data not shown).

Taken together, these data demonstrate that NS5A is able to activate Raf-1 in vivo. The NS5A-dependent activation of Raf-1, however, does not result in an activation of MEK/MAP2 kinase signalling.

Primary hepatocytes from NS5A transgenic mice are not resistant to TNFα-mediated apoptosis

To study whether the delayed elimination of LCMV in NS5A is due to a resistance of NS5A positive hepatocytes to TNFa-mediated apoptosis, we isolated primary hepatocytes from NS5A transgenic and the corresponding wt mice. The cells were treated for 24 h with TNFα (20 ng/ml) and apoptosis was analysed by western blotting

detecting PARP cleavage. The western blot (fig. 3) shows that primary hepatocytes isolated from NS5A transgenic or wt mice havea comparable sensitivity to TNFα-mediated apoptosis.

References:

Klingmuller, U., A. Bauer, et al. (2006). Syst Biol (Stevenage) **153**(6): 433-47.

Figure legends:

Figure 1:Direct interaction of Raf-1 and NS5A in vivo

a) Co-immunoprecipitation of liver-derived lysates from wild type and NS5A-transgenic mice using NS5A- or Raf-1 specific antisera. For detection a Raf-1-specific antiserum was used.

b, d, e, f) Western blot analysis of lysates derived from liver a 3 month old male mice producing NS5A or the PreS2 regulatory protein MHBst76. Sex and age matched wildtype littermates served as control. For detection a phospho-Raf (ser338) (upper panel) -and a Raf-1-specific antiserum (lower panel) (b), a phospho-MEK- (upper panel) and a MEK-specific antiserum (lower panel) (d), a phospho-MAPK- (upper panel) and a MAPK-specific antiserum (lower panel) (e) or a PCNA-specific serum (f) were used. To control equal loading an actin-specific serum was instrumental (f).

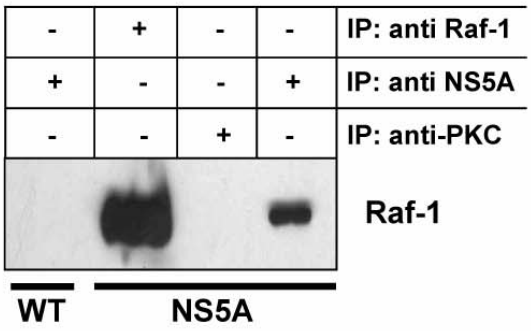
c) Raf-1 was precipitated from these lysates and Raf-1 activity was determined by immuno-complex assay (using [γ-32P]ATP and recombinant MEK as a substrate). Phosphorylated MEK (^{32}P-MEK) was visualized by SDS–PAGE and autoradiography.

Figure 2 Delayed infiltration of CD3-positive cells in LCMV-infected NS5A transgenics

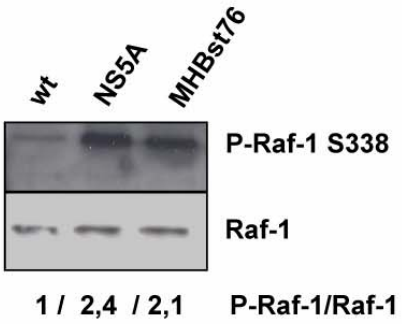
The presence of T-cells was visualized by immunohistochemistry staining of liver tissue using a CD3-specific antiserum 3, 6, 9 and 12 days after infection.

Figure 3: Primary hepatocytes from NS5A transgenic mice are not resistant to TNFα-mediated apoptosis

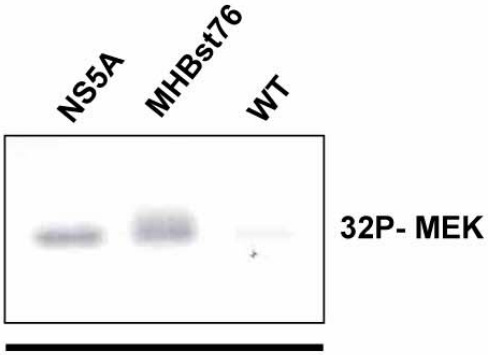
Western blot analysis of cellular lysatederived from untreated and TNFα-treated primary mouse hepatocytes derived from NS5A transgenic and wt-mice. For detection a PARP-specific antiserum was used.



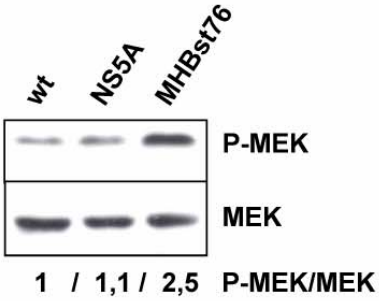
Suppl. Fig. 1a



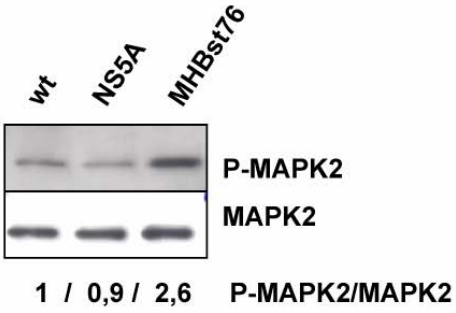
Suppl. Fig. 1b



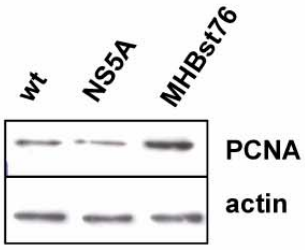
Suppl. Fig. 1c



Suppl. Fig. 1d

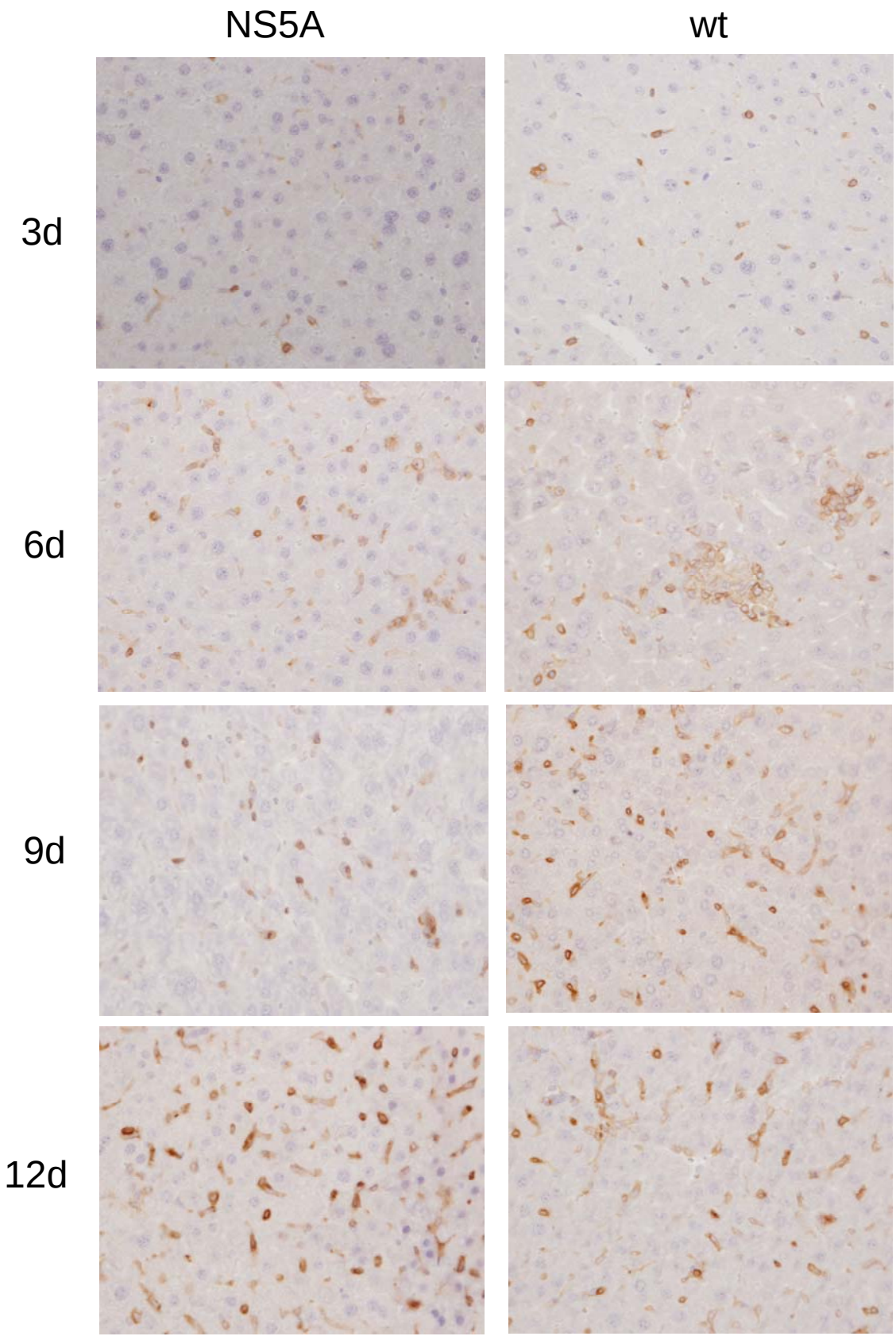


Suppl. Fig. 1e

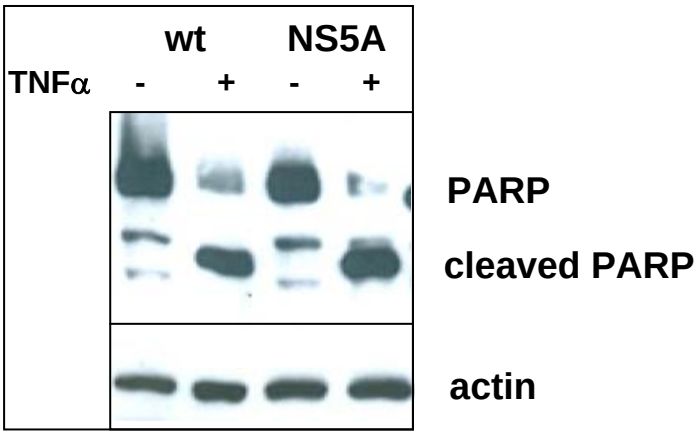


Suppl. Fig. 1f

Suppl. Fig. 1a-f



Suppl. Fig 2



Supl. Fig. 3