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LCMV-mediated hepatitis in rhesus macaques: WE but not ARM strain activates hepatocytes and induces liver regeneration

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Summary. Lymphocytic chorimeningitis virus (LCMV), the prototype arenavirus, and Lassa virus (LASV), causative agent of Lassa hemorrhagic fever (LHF), belong to the Old World group of the family *Arenaviridae*. Both viruses have extensive strain diversity and significant variations in lethality and pathogenicity for man and experimental animals. We have shown that the LHF-like infection of rhesus macaques with the WE strain of LCMV affects liver functions, induces hepatocyte proliferation, and causes a rise in IL-6 and soluble TNF receptors (sTNFR) concomitant with a rise in viremia. The levels of IL-6 and sTNFR can serve as an additional diagnostic tool for liver involvement in pathogenesis of arenavirus infection. Mucosal inoculation of rhesus macaques with LCMV-WE can result in attenuated infection with a transient viremia and liver enzyme abnormalities.

The ARM strain of LCMV shares 88% amino acid homology with WE. In contrast to LCMV-WE, ARM strain does not induce manifested disease in monkeys, does not affect liver functions, and does not induce hepatocyte proliferation. Previously we demonstrated that LCMV-ARM infection protected rhesus macaques challenged with LCMV-WE. Here we have shown that the protected animals have no signs of hepatitis and hepatocyte proliferation.

Introduction

Arenaviruses represent a fast growing group of single-stranded, two-segmented, negative-sense RNA viruses producing chronic, asymptomatic infections in their natural rodent hosts. The family *Arenaviridae* includes two phylogenetic groups of viruses: Old World and New World arenaviruses; the latter group has been further divided into A, B, and C lineages. Among all arenaviruses discovered so far six

viruses cause serious, sometimes fatal diseases in man and belong to the Old World group (isolates of the species *Lymphocytic choriomeningitis virus*, LCMV; and *Lassa virus*, LASV) and to clade B (isolates of the species *Junin virus*, JUNV; *Machupo virus*, MACV; *Guanarito virus*, GUAV; and *Sabia virus*, SABV) [3, 8, 9, 45–48, 52]. LCMV, the prototype arenavirus with world-wide distribution, induces in man a spectrum of outcomes ranging from subclinical infections to acute aseptic meningitis and congenital malformations. LASV, the etiological agent of Lassa hemorrhagic fever (LHF), causes up to 500,000 annual infections in West Africa, of which approximately 30% result in disease varying from mild influenza-like illness to lethal LHF. The burden and potential threat from LHF in endemic areas is underestimated [6]. Recent deterioration of social and economic conditions in the endemic areas increased incidence and mortality caused by LASV. This virus is also considered a potential biological weapon [7].

Arenaviruses become a public health threat when human beings contact infected rodents and/or their excreta. The natural host for LCMV is *Mus musculus* found all over the world. The natural hosts for LASV are multimammate rats (*Mastomys* spp.) widely distributed throughout sub-Saharan Africa. Arenaviruses are transmitted from rodents to humans by direct contact and/or by mucosal exposure. Population-based studies provide clear evidence that inappropriate food storage, catching, cooking and eating rats correlate with LASV infection and LHF [38, 56]. Feeding monkeys neonatal mice that were infected with LCMV caused outbreaks of hepatitis among captive callitrichid monkeys in the U.S. and in Europe [4, 43, 44, 54].

Prevention of contact between rodents and man has been most effective in controlling Bolivian HF [38]. However, vaccination is a broader and more viable control measure [6, 21, 38, 39, 46]. So far, the only arenavirus vaccine is Candid #1 against Argentine HF. This is a safe and effective live-attenuated arenaviral vaccine and application of this vaccine significantly reduced disease cases in the rural areas of Argentine [17, 18].

LASV and LCMV have extensive strain diversity with remarkable biological variations in pathogenicity and lethality [15, 24, 25, 30, 45–48]. The WE strain of LCMV is highly pathogenic for non-human primates and guinea pigs. Rhesus macaques intravenously (i.v.) infected with the WE strain of LCMV developed a severe LHF-like disease providing a valuable model for study of pathogenesis and vaccine development [48]. We have shown recently that hepatitis was one of the most prominent pathophysiological signs of the LCMV-WE infection in macaques and the infection induced strong hepatocyte proliferation [34, 35].

Here we have shown that LCMV-inducible LHF-like disease in rhesus macaques is associated with a broad functional liver damage as assessed by biochemical tests. In contrast to WE, animals infected with the ARM strain of LCMV had no manifested disease and virus was not detectable in plasma and liver. LCMV-WE-mediated liver pathology was associated with up-regulation of markers of liver regeneration: Ki-67 antigen, IL-6 and soluble TNF receptors (sTNFR). IL-6 and sTNFR in plasma were elevated after detection of virus in blood and increased rapidly during the incubation period.

Although WE is more virulent than ARM, oral infection of rhesus macaques with WE or ARM can sometimes protect animals from lethal hepatitis in challenge experiments. Protected animals had no symptoms of the disease and viremia. All liver functional tests and markers of liver regeneration were in normal ranges in the protected monkeys.

Materials and methods

Virus strains, rhesus macaque inoculations, experimental groups

WE and Armstrong 53b (ARM) strains of LCMV and virus inoculations of rhesus macaques have been previously described [32]. Fifteen healthy rhesus macaques were housed in a BSL-2/3 facility and were subsequently subdivided into two groups. Group 1 (long-term study) included five animals that have been partially described in previous publications [34, 51]. In order to compare infections with WE and ARM strains we replaced "rh" in the animal names with WE or ARM. Thus, "rh-ig7a" is "WE-ig7a" and others are "WE-ig7b", "WE-iv3", "ARM-iv3", and "ARM-ig8". Group 2 (short-term study) included ten animals inoculated with LCMV-WE (1×10^3 PFU, i.v.). One or two animals from this group were euthanized each day from day 1 untill day 7 after infection. Two animals, "Rh1a and Rh1b", were euthanized on day 1; two animals, "Rh2a and Rh2b", were euthanized on day 2; one animal, "Rh3", was euthanized on day 3; two animals, "Rh4a and Rh4b", were euthanized on day 4; two animals, "Rh6a and Rh6b", on day 6; and one animal, "Rh7", was euthanized on day 7.

Sample collections, biochemical liver tests

Blood samples were collected at specified intervals from the saphenous vein and submitted for blood counts and PBMC isolation as described [33]. Liver samples were obtained during biopsy or necropsy at the specified time and used for plaque titration to determine viral load and for RNA extraction. Plasma samples were used to assess liver function by common liver tests [12]. As markers of hepatocellular necrosis aspartate- and alanine-aminominotransferases (AST, ALT) were measured. Gamma-glutamyltransferase (GGTP), total bilirubin, and alkaline phosphatase (AP) were measured in plasma for assessment of cholestatis. Synthetic capacity of liver was evaluated by levels of albumin in plasma.

Ki-67 staining

To detect proliferating cells in liver sections immunochemical staining for Ki-67 nuclear antigen was performed as previously described [34]. Briefly, paraffin-embedded liver sections were deparaffinized, rehydrated, treated with pepsin and with Retrievit^{TM}-10 (InnoGenex, San Francisco, CA). Anti-Ki-67 antibody (Zymed Laboratories, Inc., San Francisco, CA) was used at a final dilution of 1:50. After incubating with the secondary biotinylated antibody and blocking of endogenous peroxidase (HPR) activity, streptavidin-HPR conjugate was added and signal was developed with diaminobenzidine. Cell proliferation index was determined as the fraction of cells staining for Ki-67 and expressed as a percentage of cells per field (an average of data from 10 fields). For quantitative purposes, Ki-67 control slides (Zymed #09–0040, tonsil) were included in all staining protocols and used as a standard. Positively stained liver cell nuclei were easily identifiable and clearly distinguished from background. Some variations in background staining (necropsy vs. biopsy, healthy animals vs. diseased animals) did not affect the Ki-67 proliferation index score.

Virus titration, RT/PCR, cytokine ELISAs

Detection of infectious virus in blood samples and liver extracts was performed by plaque assay on monolayers of Vero-E6 cells [33]. Viral RNA from plasma was extracted using QIAamp viral RNA mini spin protocol (Qiagen GmbH, Valencia, CA). RNA from liver tissues was extracted with Trizol (Gibco BRL, Grand Island, NY). RNA was converted to cDNA with AMV RT and random primers using a Roche Molecular Biochemicals kit (Roche Diagnostic Co., Indianapolis, IN) and analyzed using real-time PCR with SYBR green kit from Qiagen as previously described [32, 33]. The detection limit of the real-time PCR was approximately 1,000 copies of viral RNA in 1 ml of plasma.

IL-6 in plasma of rhesus macaques was measured by ELISA using OptEIA^{TM} sets (Pharmingen, San Diego, CA). The detectable IL-6 ELISA level was <5 pg/ml, IL-6 was undetectable in plasma of healthy animals. Soluble tumor necrosis factor receptors (sTNFRI and sTNFRII) were measured by ELISA kits (R&D Systems, Minneapolis, MN). In plasma of control animals, IL-6 was <5 pg/ml and levels of sTNFRI and sTNFR-II were 150–400 and 130–330 pg/ml, respectively.

Detection of anti-LCMV antibodies

Antibody responses were measured by ELISA and neutralization assays. Viral antigen for ELISA was prepared from serum-free culture medium of Vero cells as described previously [34]. 100 µl of viral antigen in carbonate-bicarbonate buffer was used to cover wells of microtitration plates and incubated overnight at 4 °C. After blocking with 10% FCS in PBS, ten-fold dilutions of monkey sera were added and incubated for 2 h at room temperature. Peroxidase-labeled goat anti-monkey IgG (KPL, Gaithersburg, MD) was used at a final dilution of 1:2000 and substrate solution (Turbo TMB-ELISA, Pierce, Rockford, IL) was added for color development. Neutralization antibody titers were measured by plaque reduction neutralization (PRNT) assay using a constant dose of virus, Vero cell monolayers and serial 1-log dilutions of plasma. Incubation of virus with serum was performed at 37 °C for 1 h. As a control, serum collected before infection was used. End points were calculated from the highest serum dilution inducing 50% plaque reduction.

Challenge experiments

The surviving animals from group 1 (WE-ig7a, WE-ig7b, ARM-iv3, and ARM-ig8) were challenged with 1×10^3 PFU of LCMV-WE (i.v.). Two WE-infected monkeys, WE-ig7a and WE-ig7b, were challenged on day 98 and 112 after the primary infection, respectively. Two ARM-infected monkeys, ARM-iv3 and ARM-ig8, were challenged on day 56 after the primary infection. After challenge animals were observed up to 10 weeks and blood samples were collected at 2 week intervals. Liver biopsy samples were collected at 4 week intervals. Hepatitis development in challenged animals was evaluated by biochemical liver tests and by measurement of hepatocyte proliferation markers (Ki-67 staining, IL-6, sTNFRI and sTNFRII).

Cell-mediated immunity

CTL and lymphocyte proliferation assays followed previously published protocols [14, 51, 61]. In brief, effector cells (PBMC from infected monkeys) were cultivated in RPMI 1640 with 20% FBS, concanavalin A (Con A) and IL-12 for 3 days, washed and cultivated again for 7 days without Con A. Target cells (autologous B-lymphoblastoid cell lines established by transformation of monkey PBMC with *Herpes papio*) were infected with recombinant vaccinia (rVV) expressing nucleocapsid (NP) or glycoprotein (GP) genes of LCMV, rVV-NP, rVV-GP [58]. ^{51}Cr-labeled targets cells (2×10^4) were plated in 96-well plates in triplicate

for each effector:target ratio, 50:1, 25:1, and 12.5:1. Specific lysis was calculated as follows: [(experimental lysis – spontaneous lysis)/(maximum lysis – spontaneous lysis)] × 100. Spontaneous lysis varied from 10 to 20% and specific lysis was considered positive if it was above mean and standard deviation of background lysis using uninfected or VVwt-infected targets.

For proliferation assay [14] fresh PBMC (10^5 in 100 μl) were plated in triplicate for each of the different stimuli in 96-well plates. Each well received an additional 100 μl of RPMI 1640 (negative control), RPMI + PHA, 10 μg/ml (mitogen control) or RPMI + inactivated LCMV (WE or ARM, 3×10^5 PFU before inactivation). Plates were set up in duplicate, incubated for 3 days, pulsed with ^{3}H-thymidine and supernatants were harvested 12 hours after labeling. The stimulation index (SI) was determined as the mean number of ^{3}H cpm (counts per minute) incorporated in the presence of LCMV antigen divided by the mean cpm incorporated in the presence of medium control. The SI > 5 was considered as specific response.

Data analysis

Statistical analyses and graphing were performed using the Origin 6.0 package (Microcal Software, Inc., Northampton, MA).

Results

Outcome of LCMV infection of rhesus macaques after primary infection and after challenge

From five monkeys infected with LCMV (Table 1), three animals, WE-ig7a, ARM-iv3, and ARM-ig8, showed no signs of disease. From two diseased animals, WE-iv3 and WE-ig7b, rhesus WE-iv3 had LHF-like manifestations and died on day 11. After necropsy the highest viral titers were found in liver, blood, and spleen. This monkey was a "positive" control for the LCMV-WE viral challenge stock [33].

Rhesus WE-ig7b became ill at 21 days after infection and developed a transient fever, weight loss and viremia. Liver biopsy samples taken at week 4 after infection were negative for LCMV antigens by immunohistochemistry but gave strong positive RT/PCR signals with primers to the LCMV-WE GP and NP genes (not shown). The disease lasted for two weeks, followed by full recovery associated with development of strong immune responses (Table 1 and Ref. [51]). Before the challenge anti-LCMV antibodies reached 7.2 and 3.3 $\log_{10}$ as detected by ELISA and plaque neutralization assay, respectively. PBMC from this monkey showed 47% and 64% specific lysis against targets infected with VV-NP and VV-GP, respectively (Table 1).

Rhesus ARM-iv3 and ARM-ig8 did not develop clinical symptoms of the disease and the virus was not detected by RT/PCR in any tested plasma and liver biopsy samples. Asymptomatic ARM infection in these animals was associated with development of immune responses. At 10 weeks after the primary infection titers of anti-LCMV IgG antibodies detected by ELISA were 5.4 and 5.8 $\log_{10}$ for rhesus ARM-iv3 and ARM-ig8, respectively. Low titer neutralizing antibodies were found only in i.v.-inoculated monkey, ARM-iv3 (Table 1). Circulating PBMC isolated from ARM-infected animals developed proliferation responses after incubation with specific antigens. Rhesus WE-ig7a did not develop manifested disease and virological (plaque assay, RT/PCR) and serological

Table 1. Main characteristics of rhesus macaques infected and challenged with LCMV

Characteristic	Monkey*				
	WE-iv3	WE-ig7a	WE-ig7b	ARM-iv3	ARM-ig8
*Primary infection***					
Outcome	D11	S	S	S	S
Hepatitis	+	−	+/−	−	−
Ki-67	+	−	+/−	−	−
IL-6/sTNFRs	+	−	+/−	−	−
Viremia	+	−	+/−	−	−
IgG ELISA	<2	<2	7.2	5.4	5.8
PRNT	<1	<1	3.3	1.2	<1
SI	ND	<5	25/12	13/90	7/18
CTL (% Spec. Lysis)	ND	17/21(10)	47/64(8)	ND	ND
*Challenge***					
Outcome		D14	S	S	S
Hepatitis		+	−	−	−
Ki-67		+	−	−	−
IL-6, sTNFRs		+	−	−	−
Viremia		+	−	−	−
IgG ELISA		<2	6.0	5.2	5.3
PRNT		<1	2.0	1.9	<1
SI		ND	50/8	19/82	3/15
CTL (% Spec. Lysis)		5/12(6)	47/44(8)	ND	ND

*Rhesus macaques were infected with WE or ARM strains of LCMV using intravenous (i.v.) or intragastrical (i.g.) routes. Rhesus WE-iv3 received 1×10^3 PFU of WE strain and died on day 11 after infection. Two monkeys, WE-ig7a and WE-7b, were i.g. inoculated with 1×10^7 PFU of LCMV-WE, and two monkeys, ARM-iv3 and ARM-ig8, were infected with LCMV-ARM, 1×10^3 PFU and 1×10^8 PFU using i.v. and i.g. routes, respectively

**"Outcome", D11, death at day 11 after infection. S, animal survived. "Hepatitis" was evaluated by biochemical liver tests and marked as "+" if liver tests were elevated in be-weekly bleeding samples; "+/−", transient elevation of liver enzymes on week 4 after infection; "−" no biochemical signs of hepatitis. "Ki-67+", positive staining on Ki-67 antigen of monthly collected liver biopsy samples or necropsy samples (more than 5% of positive nuclei); "+/−", transient positive staining at week 4 after infection; "−", less than 5% positively stained nuclei. "IL6/sTNFR+", detection of IL-6 in plasma and levels of sTNFRI and RII higher than detectable levels (3.0 and 6.5 pg/ml, respectively) in bi-weekly collected plasma samples; "+/−" transient IL-6 detection and sTNFR elevation, at week 4 after infection; "−", no IL-6, sTNFRs below detectable levels. "Viremia+", detection of the virus in plasma; "+/−", transient viremia in plasma samples collected at 4 weeks after infection; "−", viremia below detectable level, 1.3 $\log_{10}$ PFU/ml. IgG ELISA and plaque neutralization (PRNT) titers expressed as $\log_{10}$ dilutions. For primary infection animals antibody titers are indicated at day of the challenge, 98 and 112 days for rhesus WE-ig7a and WE-ig7b, respectively, and 56 days for ARM-infected monkeys, ARM-iv3 and ARM-ig8. For challenged animals titers are shown at the week 10 after challenge. "SI", stimulation index evaluated in lymphocyte proliferation assay and expressed as the mean number of radioactivity incorporated in the presence of autologous and heterologous antigens, WE/ARM (see Methods). The highest levels

(ELISA, PRNT) tests were negative. However, at 10 weeks after infection low but reproducible LCMV-specific cytotoxic activities against targets expressing NP and GP antigens were found in this monkey (Table 1).

Animals surviving after the primary infection were challenged with LCMV-WE using lethal i.v. inoculation (1×10^3 PFU). As seen from Table 1, rhesus WE-ig7a developed fever and viremia and died on day 14 with hemorrhagic and hepatic manifestations similar to those found in rhesus WE-iv3. Rhesus monkeys WE-ig7b, ARM-iv3, and ARM-ig8 survived after challenge. The surviving animals did not develop fever or any other symptoms of the disease. Protection of these animals seems to be not associated with induction of neutralizing antibodies and correlated with cell-mediated immunity (Table 1).

LCMV-WE but not LCMV-ARM affects liver functions in rhesus macaques

Postmortem histological studies of LHF patients and monkeys infected with LASV or LCMV-WE showed that the most important pathological changes are hepatic and that liver disease was a necessary, but not sufficient, condition in the events leading to death [33–35, 41, 46–48, 57]. As seen in Fig. 1, on the day of death levels of aminotransferases, AP, GGTP and bilirubin were significantly elevated in the fatally infected WE-iv3 monkey indicating the development of hepatocellular necrosis and cholestasis. Levels of albumin in plasma deeply dropped after the infection. In plasma samples collected from monkeys involved in a short-term study these liver abnormalities were found only in Rh-6a, Rh6-b and Rh-7 animals (not shown).

In the diseased WE-ig7b animal (long-term study) liver tests showed transient abnormalities. Plasma levels of ALT, AST, GGTP, AP, and bilirubin were elevated on 28–42 days after infection and reversed to normal ranges in 2–3 weeks. Albumin level dropped 2-fold at week 4 and reversed to the normal ranges in 2 months. In WE-ig7a monkey (without apparent disease) and in rhesus macaques infected with ARM strain of LCMV most biochemical liver tests were within the normal ranges.

WE-infection but not ARM-infection is associated with markers of hepatocyte activation

Histopathological studies performed by McCormick et al. [41] provided clear evidence of hepatocellular mitosis in LHF patients. Recently we have found evidence of hepatocyte proliferation in rhesus macaques with fatal LHF-like disease [33].

proliferative responses are shown in the table, 4 weeks after primary infection and 4 weeks after challenge. “CTL”, cytotoxic T lymphocyte assay (see Methods for details). Specific lysis values are shown against targets expressing NP and GP antigens, NP/GP. Although the assays were performed at effector: target ratios of 50:1, 25:1, and 12.5:1, only results from the 50:1 ratio are shown. The highest levels of CTL are shown in the table, time points after primary and challenge infection are indicated in brackets. “ND”, not done

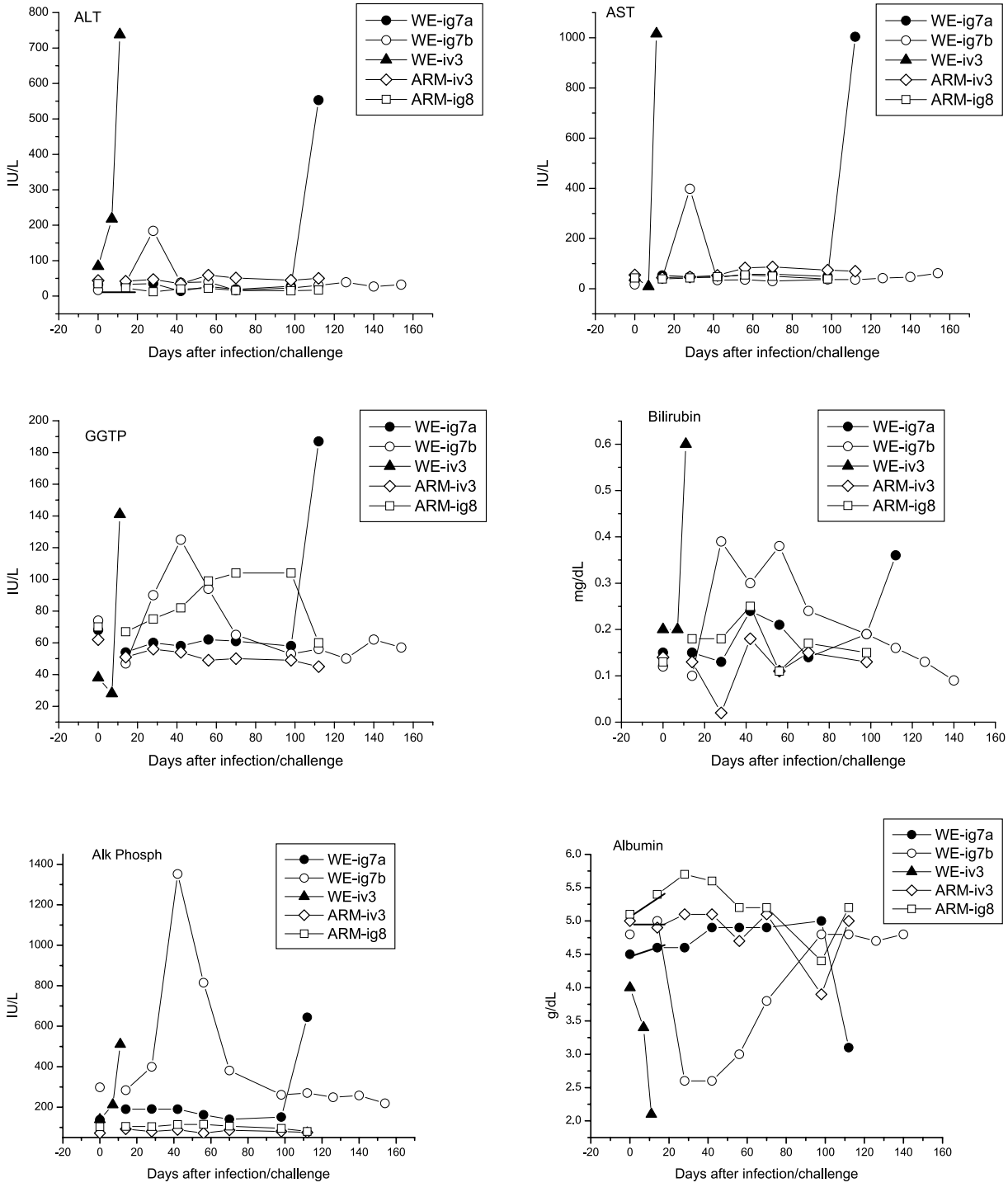


Fig. 1. Biochemical liver abnormalities in LCMV-infected rhesus macaques. Markers of hepatocellular necrosis (AST, ALT), cholestatis (GGTP, bilirubin, alkaline phosphatase), and hepatic synthetic activity (albumin) were measured in plasma samples collected from WE- and ARM-infected monkeys. References ranges are 18–82, 22–87, 28–86 IU/l for AST, ALT, and GGTP, respectively; 0.1−<0.3 mg/dl, 30–120 IU/l, and 4.0–6.0 g/dl for bilirubin, alkaline phosphatase and albumin, respectively [12]

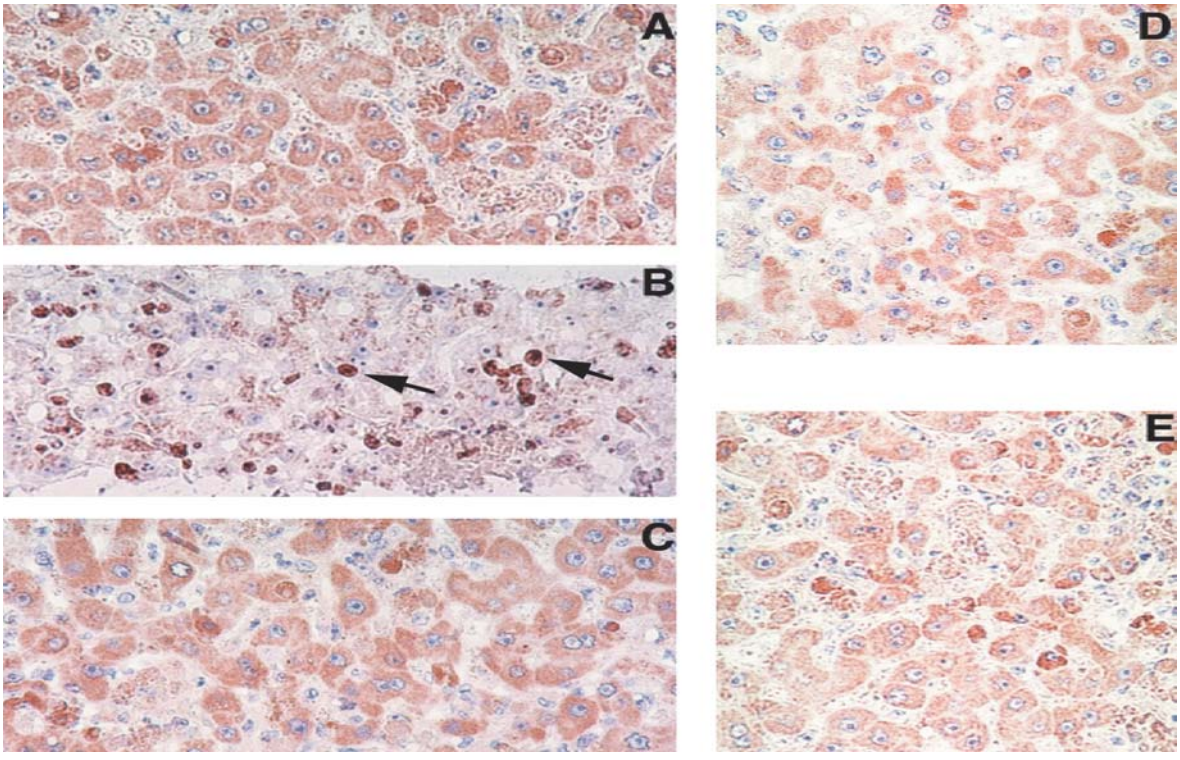


Fig. 2. Ki-67 staining of liver sections from rhesus macaques infected with LCMV. **A**, **B**, **C**: Liver biopsies taken from monkey WE-ig7b before infection (**A**), four weeks after infection (**B**), and four weeks after challenge (**C**). **D** and **E**: Ki-67 staining of biopsy liver samples taken from ARM-iv3 (**D**) and ARM-ig8 (**E**) monkeys at four weeks after infection. Magnifications: ×300. Note brown staining of Ki-67-positive nuclei (arrowed) in **B**

As seen in Fig. 2, nuclei of rhesus WE-ig7b hepatocytes were positively stained for proliferation Ki-67 antigen. The cell proliferation index before infection was less than 1% and reached at least 25% for biopsy samples taken at the peak of the disease (4 weeks post infection). A month later the number of positively stained nuclei reversed to the background level. In ARM-infected animals the proliferation index was 1–2% before and after infection.

A large body of evidence, primarily from murine studies, indicates that IL-6 and sTNFR plays a key role in hepatocyte proliferation after surgery/toxic injury [2, 10, 19, 22, 28, 37, 42, 59, 60, 62]. Virus-mediated hepatocyte proliferation seems to exploit a similar pathway [34, 35, 41, 63]. To evaluate the diagnostic value of IL-6 and soluble TNF receptors in LCMV-inducible liver pathology in rhesus macaques, plasma levels of these markers were measured in two groups of animals, group 1 (long-term study) and group 2 (short-term study). In healthy rhesus macaques IL-6 is not detectable in plasma. In the WE-iv3 animal the level of IL-6 reached 780 pg/ml at the day of death and plasma levels of sTNFRI and sTNFRII were elevated more than 5–10-fold over pre-infection levels (Fig. 3).

In rhesus WE-ig7b, the monkey that recovered from i.g. infection, IL-6, sTNFRI and TNFRII were elevated in parallel with viremia, peaking at 4–5 weeks

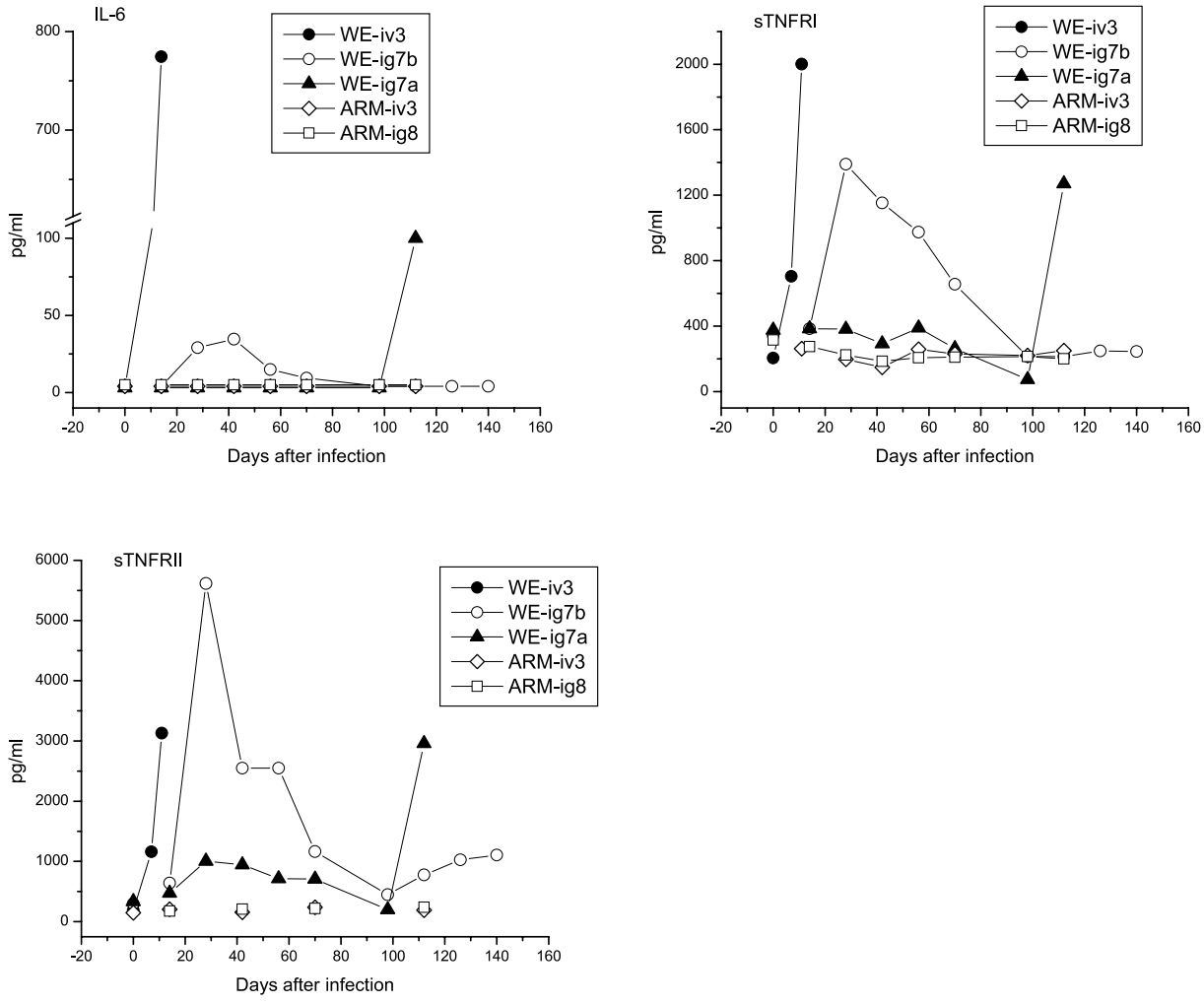
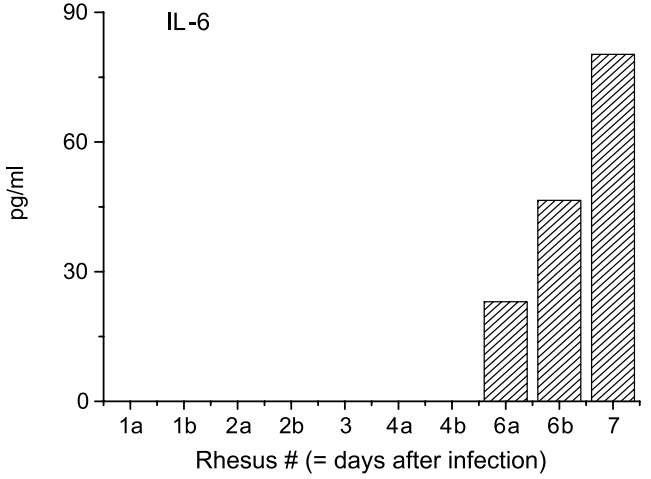
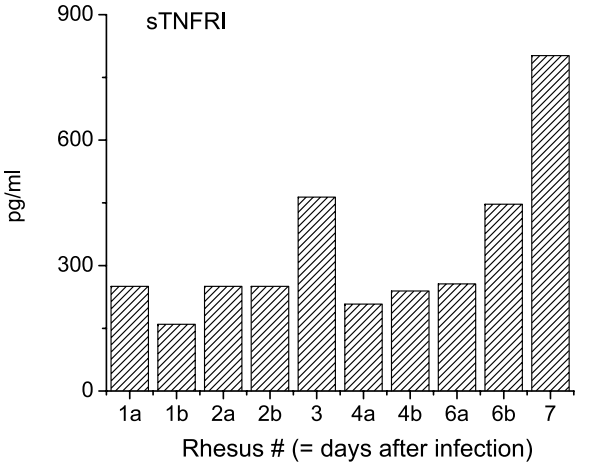
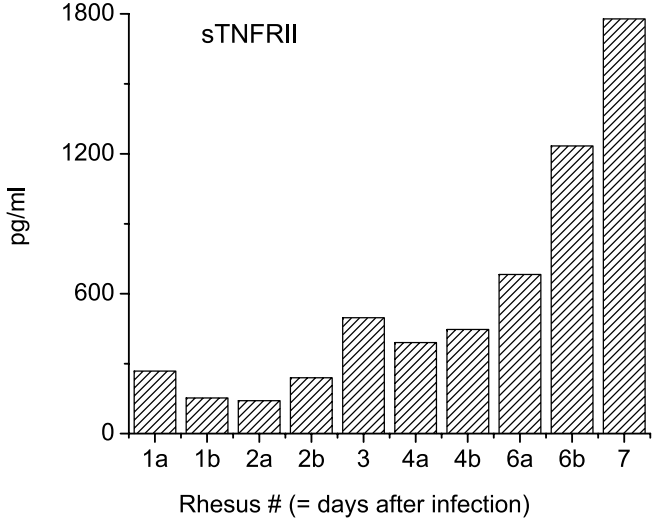


Fig. 3. IL-6 and sTNFRs in plasma of LCMV-infected monkeys before and after challenge. Rhesus macaques were infected and challenged as described in "Materials and methods". Plasma samples were assayed in triplicate and expressed as mean values. The minimum detectable doses were <5.0, 3.0, and 6.5 pg/ml for IL-6, sTNFRI, and sTNFRII, respectively

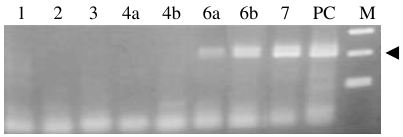
Fig. 4. Viremia, IL-6 and soluble TNF receptors at the early stage of LCMV-WE-infection Ten rhesus macaques were i.v. inoculated with 1×10^3 PFU of LCMV-WE and euthanized at day 1 (animals Rh-1a, b); 2 (Rh-2a, b); 3 (Rh-3); 4 (Rh-4a, b); 6 (Rh-6a, b); and day 7 (Rh-7). IL-6 and sTNFRI and II were measured in plasma as described in "Materials and methods". Viral RNA from plasma was extracted, converted into cDNA and quantitative PCR with GP primers 1–19 and 499–480 (499 bp PCR product) was performed as previously described [33]. Virus was recovered by cultivation on Vero E6 cells and by plaque assay. PC, positive control, viral RNA from plasma of rhesus WE-iv3







WE RT/PCR



Samples	Ct	Virus recovery
1–3	> 38.0	-
4a	37.63+/-0.48	-
4b	34.86+/-0.13	+
6a	30.46+/-0.26	+
6b	30.73+/-0.29	+
7	25.14+/-0.06	+
PC(11d)	23.84+/-0.42	7.2 log 10 PFU/ml

after infection and gradually dropping to undetectable levels (IL-6) or to the normal ranges (sTNFRI and II) in 3 months (Fig. 3). In WE-ig7a (no manifested infection) the measured markers of hepatocyte proliferation were in the normal ranges. In ARM-infected monkeys the infection did not induce detectable levels of IL-6. Plasma levels of sTNFRs were in the normal ranges during the whole observation period.

In a short-term study plasma samples were collected to measure viral RNA load, infectivity, levels of IL-6 and sTNFRs. Semi-quantitative PCR studies showed that LCMV-WE viral RNA in plasma was detected as early as day 4 after infection. As seen in Fig. 4, RNA samples from plasma of monkey Rh-4b had $C_t < 35$ in semi-quantitative PCR and specificity of PCR was confirmed by nested PCR (not shown). The virus was also recovered from these samples by biological amplification in tissue culture. For monkey Rh-4a the C_t value was well below the detection limit (>37) and the virus was not recovered in tissue culture. The end of incubation period and the appearance of the first clinical symptoms [33] were associated with rapid accumulation of viral RNA and infectious virus in plasma leading to the lethal outcome on day 11 (Fig. 4). The plasma viremia on the day of death was more than 7 $\log_{10}$ PFU/ml.

IL-6 in plasma of animals from group 2 (a short-term study) was detected on day 6 and increased until 80 pg/ml at day 7. Concentrations of sTNFRI and II were elevated at day 6 after infection. Marginal elevation of these receptors was also noted on day 3. On day 6 and 7, levels of these receptors were 2–4-fold over pre-infection ranges.

Vaccinated rhesus macaques protected against lethal challenge had no hepatitis and hepatocyte activation

In a monkey that survived after lethal challenge (rhesus WE-ig7b, ARM-iv3, and ARM-ig8) all liver tests were in the normal ranges (Figs. 1, 2). IL-6 in plasma was not detectable and levels of soluble TNF receptors were also in the normal ranges. Marginal elevation of sTNFRII was observed in the WE-ig7b monkey. Ki-67 staining of WE-ig7b liver biopsy samples taken at 4 weeks after challenge revealed no more than 1–2% positive nuclei. Liver biopsy samples taken from ARM-vaccinated animals, ARM-iv3 and arARM-ig8, were also negative on Ki-67 nuclear antigen.

Monkey WE-ig7a was not protected against WE challenge and died on day 14 with LHF manifestations. The animal had viremia, virus was detected in liver and all measured markers of liver function, ALT, AST, GGTP, total bilirubin, and alkaline phosphatase were significantly elevated. The level of plasma albumin was lowered (Fig. 1). Markers of hepatocyte proliferation, IL-6, sTNFRI, II (Fig. 2) and Ki-67 (not shown) were strongly elevated in this animal.

Discussion

Postmortem histological studies of LHF patients and LASV-infected experimental animals indicate that the liver is one of the most affected organs participating in a

systemic breakdown [16, 20, 24–27, 38–41, 48, 57]. Three categories of morphological changes were found in liver of fatal LHF patients: active hepatocellular injury, continued damage and early recovery, and regenerative phase with high mitotic activity of hepatocytes [41]. Despite the substantial liver dysfunction, there were few indications of microscopic pathology in livers from LHF patients and experimentally infected animals. The relationship between the liver pathology, hematological and endothelial dysfunctions leading to shock and death remains unclear.

Rhesus macaques inoculated with LCMV-WE rapidly develop a fatal LHF-like disease providing a valuable model for human LHF [24, 33, 48]. We have observed substantial hepatocyte proliferation in both LCMV i.v.- and i.g.-inoculated rhesus macaques and have shown that during peak disease at least 25–40% of hepatocyte nuclei in liver biopsy or necropsy samples stained positively for proliferation antigen Ki-67 [34]. In this communication we have shown that the WE, but not the ARM strain of LCMV, affects biochemical, excretory, and perhaps synthetic functions of liver and that only the WE strain induces hepatocyte proliferation accompanied by high levels of IL-6 and sTNFRs in plasma.

LASV infection elevates hepatic aminotransferase levels that seem to correlate with viremia and disease progression in man and in experimental animals [24, 25, 30, 34, 35, 40, 41]. Commonly used tests for evaluation of excretory liver functions include total bilirubin, GGTP, and alkaline phosphatase [12]. We have found elevation of these tests in the WE-infected monkeys (Fig. 1). Clinically no jaundice was observed in LHF patients and bilirubin levels were near normal [41]. In contrast, callitrichid hepatitis in New World monkeys caused by LCMV-CH was associated with jaundice, extremely high levels of GGTP and bilirubin in plasma [43, 44, 53].

Albumin plasma levels dropped in fatally infected LCMV-WE monkeys (Fig. 1). No significant renal failure was mentioned as judged by levels of urea nitrogen and creatinine (not shown). In rhesus WE-ig7b the initial stage of the disease was also associated with decreased concentrations of albumin in plasma. However, during the recovery stage the albumin concentration increased to normal ranges. In fatally infected monkeys endothelial dysfunction seems to be an additional factor contributing to albumin decrease.

The first observation of hepatocellular mitosis in LHF patients was provided by McCormick et al. [41] using H&E staining of liver samples taken within 1–2 hrs after death. As it has been mentioned, LCMV-WE induces a LHF-like disease in rhesus macaques. Using liver biopsy and necropsy samples from WE-infected monkeys we have found that 25–40% of hepatocyte nuclei from liver of LCMV-WE-infected rhesus macaques were positively stained for Ki-67 [34, 35]. Here we showed that recovery from the disease was accompanied by disappearance of Ki-67 staining. Successfully vaccinated and protected monkeys showed no increase in Ki-67 staining after challenge with LCMV-WE (Fig. 2). It suggests that hepatocyte proliferation is marker of the disease in this model.

Among various growth factors and cytokines, IL-6 and its signaling cascade through STAT3, are both important for liver regeneration and for hematopoietic

cell proliferation [2, 10, 19, 23, 29, 37, 49, 55]. Severe bone marrow depletion and thrombocytopenia were found in LCMV-WE-infected monkeys [33]. Thrombocytopenia and platelet function depression were also involved in pathogenesis of human LHF [11, 20]. It is possible that IL-6 and its signaling are playing a role in hematopoiesis reparation.

TNF-α and its receptors are other important players in initiation of liver regeneration [1, 19, 42, 62]. Interestingly, Mahanty et al. [36] have found that the levels of sTNFRs were significantly higher in patients with fatal LHF than in those with a less severe disease. In HCV patients, levels of sTNFRs did not correlate with TNF-α, but do correlate well with aminotransferase levels [63]. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C viral infection revealed the most notable changes in numerous interferon response genes. In addition, IL6R and TNFR genes were upregulated 5–8 fold [5].

We have not found elevation of TNF-α in plasma of LCMV-WE infected monkeys [34]. It confirms data on LHF patients [36] and our previously published results on human monocytes/macrophages and endothelial cells infected with LASV [32]. Levels of sTNFRs have been elevated in LCMV-WE infected rhesus macaques (Fig. 3). It is not clear whether elevated levels of sTNFRs reflect overexpression or enhanced shedding of these receptors or both. Experiments on mice lacking sTNFRI demonstrated the importance of this type of receptor in liver regeneration [19, 59, 60]. Recently Xia et al. [70] have found that liver regeneration was associated with apoptosis of hepatocytes which is mediated by sTNFRI and proposed that membrane-anchored metalloproteases could play a role in shedding membranous TNFRI.

Results from our short-term study demonstrate that IL-6 and sTNFRs elevation is an early event following viremia (Fig. 4). In contrast to sTNFRs, IL-6 is not detectable in plasma of naïve, healthy rhesus macaques. This suggests that detection of IL-6 in plasma is an accurate pathophysiological marker of liver involvement at the early stage of the disease.

One of the main conclusions of this study is that the infection with two LCMV strains, WE and ARM, has opposite influence on liver pathology in rhesus macaques. These strains also differ in their ability to cause a lethal disease in guinea pigs. Reassortant studies mapped pathogenic potential to the larger (L) of the two viral genomic RNA segments [31, 50]. We have completed the nucleotide sequences for both the WE and ARM strains of LCMV and have found them to be 84% homologous (L RNA) [13, 52]. At the amino acid level similarity between the two viruses is even higher, 87–88% (for Z and L proteins). The most divergent regions were found in the N-terminal parts of the L and Z proteins and these regions are most likely to account for differences in pathogenic potential [13].

Our working hypothesis is that the virus must first replicate to a certain level before tropism and other factors begin to affect virulence. The highest level of replication of LASV and LCMV-WE (but not ARM) was found in liver tissues. Preliminary *in vitro* results have shown that the WE strain of LCMV replicates more efficiently in primary monkey hepatocytes in comparison with

the ARM strain (Lukashevich et al., unpublished observations). Host and viral factors responsible for high replication of WE in liver tissues and mechanisms of virus-inducible hepatocyte proliferation remain to be elucidated.

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