Mouse Hepatitis Virus Infection Induces a TLR2-Dependent Activation of Inflammatory Functions in Liver Sinusoidal Endothelial Cells during Acute Hepatitis

Christian Bleau, Aveline Filliol, Michel Samson, Lucie Lamontagne

To cite this version:
Christian Bleau, Aveline Filliol, Michel Samson, Lucie Lamontagne. Mouse Hepatitis Virus Infection Induces a TLR2-Dependent Activation of Inflammatory Functions in Liver Sinusoidal Endothelial Cells during Acute Hepatitis. Journal of Virology, American Society for Microbiology, 2016, 90 (20), pp.9096–9113. <10.1128/JVI.01069-16>. <hal-01390974>

HAL Id: hal-01390974
https://hal-univ-rennes1.archives-ouvertes.fr/hal-01390974
Submitted on 2 Nov 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
MOUSE HEPATITIS VIRUS INFECTION INDUCES A TLR2-DEPENDENT ACTIVATION OF INFLAMMATORY FUNCTIONS IN LIVER SINUSOIDAL ENDOTHELIAL CELLS DURING ACUTE HEPATITIS

Christian Bleau\textsuperscript{1}, Aveline Filliol\textsuperscript{2}, Michel Samson\textsuperscript{2}, and Lucie Lamontagne\textsuperscript{1}

\textsuperscript{1}Department of Biological Sciences, Université du Québec à Montréal, Montreal Canada, H3C 3P8; \textsuperscript{2}U.1085 Inserm, IRSET, Institut de recherche en Santé-Environnement-Travail, Université de Rennes 1, 35043 Rennes, France

Corresponding author

Dre Lucie Lamontagne DMV, Ph.D
Département Sciences Biologiques
Université du Québec à Montréal,
C.P. 8888 Succ. Centre-Ville
Montréal, Qué.
H3C 3P8
Tel : (514) 987-3000 poste 3184
Fax : (514) 987-4647
e-mail : lamontagne.lucie@uqam.ca

Running title: MHV3 reverts liver sinusoidal endothelial cell functions

Word count for the abstract: 254
Word count for the text: 8071

Key words: liver sinusoidal endothelial cell, mouse hepatitis virus, inflammation, tolerance, cytokines, chemokines, Fgl2, IL-33.
ABSTRACT

Under physiological conditions, the liver sinusoidal endothelial cells (LSECs) mediate hepatic immune tolerance towards self or foreign antigens through constitutive expression of anti-inflammatory mediators. However, upon viral infection or TLR2 activation, LSECs can achieve proinflammatory functions but their role in hepatic inflammation during acute viral hepatitis is unknown. Using the highly virulent mouse hepatitis virus (MHV) type 3 and the attenuated variants 51.6-MHV3 and YAC-MHV3, exhibiting lower tropism for LSECs, we investigated *in vivo* and *in vitro* the consequence of LSEC infection on their pro-inflammatory profile and the aggravation of acute hepatitis process. *In vivo* infection with virulent MHV3, in comparison to attenuated strains, resulted in fulminant hepatitis associated with higher hepatic viral load, tissue necrosis, levels of inflammatory mediators and earlier recruitment of inflammatory cells. Such hepatic inflammatory disorders correlated with disturbed production of IL-10 and vascular factors by LSECs. We next showed *in vitro* that infection of LSECs by the virulent MHV3 strain altered their production of anti-inflammatory cytokines and promoted higher release of pro-inflammatory and procoagulant factors and earlier cell damage in comparison to attenuated strains. This higher replication and pro-inflammatory activation in LSECs by the virulent MHV3 strain was associated with a specific activation of TLR2 signalling by the virus. We provided evidence that TLR2 activation of LSECs by MHV3 is an aggravating factor of hepatic inflammation and correlates with the severity of hepatitis. Taken together, these results indicate that preservation of immunotolerant properties of LSECs during acute viral hepatitis is an imperative factor to limit hepatic inflammation and damages.
IMPORTANCE

Viral hepatitis B and C infections are serious health problems infecting over 350 million and 170 million people worldwide respectively. It has been suggested that a balance between protection and liver damage mediated by the host’s immune response during the acute phase of infection would be determinant in hepatitis outcome. Thus, it appears crucial to identify the factors that predispose in exacerbating liver inflammation to limit hepatocyte injury. Liver sinusoidal endothelial cells (LSECs) can express both anti- and pro-inflammatory functions but their role in acute viral hepatitis has never been investigated. Using the mouse hepatitis virus (MHV) infections as animal models of viral hepatitis, we report for the first time that in vitro and in vivo infection of LSECs by the pathogenic MHV3 serotype leads to a reversion of their intrinsic anti-inflammatory phenotype towards a pro-inflammatory profile as well as disorders in vascular factors, correlating with the severity of hepatitis. These results highlight a new viral-promoted mechanism of exacerbation of liver inflammatory response during acute hepatitis.
INTRODUCTION

Under physiological conditions, the liver adopts mechanisms of immune tolerance towards innocuous gut-derived food and microbial antigens (such as LPS) to prevent undesired inflammatory responses. The induction of tolerance in the liver is mediated by several resident hepatic cells such as the endothelial cells lining the hepatic sinusoids (LSEC), the Kupffer cells (KC) and in a lesser extent the hepatocytes (1). However, LSECs tolerizing and anti-inflammatory functions were recently shown to be more efficient than those of KCs (2). Given their anatomical situation, LSECs are first in contact with portal-delivered antigens and thus act as a sieving barrier in expressing highly efficient sentinel and scavenger functions that contribute to clearance of microbial products (3). They also tightly control blood-parenchyma exchanges via a dynamic regulation of the sinusoidal blood flow in releasing vasoactive factors such as NO (reviewed in 4). LSECs play a major role in liver tolerance in displaying a restricted toll like receptor (TLR)-mediated activation profile to microbial products (5, 6) and producing high amounts of anti-inflammatory cytokines, such as tumor growth factor (TGF)-β and IL-10 (7, 8). However, upon viral infection or stimulation by TLR1/2 ligands, LSECs can switch towards an inflammatory and immunogenic state and induce recruitment of leucocytes and virus-specific CD8+ T cell immunity (5, 9). The role of LSECs in inflammatory liver diseases is poorly known but as these cells can express both anti- and pro-inflammatory functions, they could act as moderator or rather exacerbator of liver inflammation.
Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems affecting over 350 million and 170 million people worldwide respectively (10). Most liver damages in HBV/HCV infections are primarily attributed to an exuberant immunopathological response triggered by viral infection rather than direct injury caused by viral replication (11, 12). It has been suggested that the balance between protection and liver damage mediated by the host’s immune response during the acute phase of infection would be critical in the outcome of hepatitis (13). Evidence suggests that an exacerbated hepatic inflammatory response during acute infection may predispose to the development of a fulminant hepatic failure characterized by extensive hepatocellular dysfunctions and high mortality (14). The role of LSECs in viral hepatitis is largely unknown and data are somewhat contradictory. Indeed, LSECs were suggested to contribute to the clearance of HCV and HBV from the bloodstream (15, 16) and to control HCV replication (17) or rather promote its transmission to hepatocytes in acting as a viral reservoir (18). Few data suggest that LSECs may also participate to hepatic inflammation since the fibrinogen-like factor 2 (Fgl-2), promoting vascular thrombosis and hepatic inflammation, and the proinflammatory alarmin IL-33, both produced by LSECs, are up-regulated in acute or chronic hepatitis (19-22). A better understanding of the role of LSECs during the acute phase of viral hepatitis may help to identify new mechanisms that predispose to inflammation-driven hepatocyte injury and liver failure.

The mouse hepatitis virus type 3 (MHV3), belonging to coronavirus family, is a relevant murine model to unravel the role of LSECs in acute viral hepatitis. MHV3 infects LSECs,
hepatocytes, Kupffer (KC) and Ito cells all expressing the carcinoembryonic antigen 1a (CEACAM1a) viral receptor and induces fulminant hepatitis leading to death of susceptible C57BL/6 mice within 3–4 days (23-25). LSECs are thought to play an important role in the resistance of A/J mice to MHV3 infection in controlling viral replication and delaying the transmission of the viral progeny to hepatocytes (26). Previous studies have reported early structural and vascular disorders in LSECs during MHV3 infection in susceptible C57Bl/6 mice. Indeed, a reduced number of fenestrations in liver sinusoids and a correlation between the fulminance of hepatitis and the induction of Fgl2 in LSECs have been described (27, 28). We have also shown that MHV3 infection was associated with early release of IL-33 by LSECs (29) and a reduction in the intrahepatic levels of immunosuppressive IL-10, PGE2 and TGF-β cytokines suggesting viral-induced disturbances in LSEC-mediated liver tolerance (30). Several attenuated MHV3 variants, such as the 51.6- and YAC-MHV3 viruses, have been in vitro generated to study the role of specific hepatic cells in hepatitis process. Compared to the parental MHV3, the major difference of the 51.6-MHV3 variant is its inefficiency to replicate in LSECs (24). Such difference is reflected by induction of milder hepatitis and higher hepatic levels of IL-10 and TGF-β (30). The YAC-MHV3 variant, showing lower tropism for LSECs and macrophages, induces a subclinical hepatitis characterized by few perivascular inflammatory foci (31) and higher induction of anti-inflammatory cytokines in the liver than 51.6-MHV3 (30). The absence of vascular thrombosis combined with efficient recruitment of mononuclear cells favor hepatic clearance of YAC-MHV3 and full recovery of infected mice within 15 days (32). These improved clinical outcomes in mice infected by the attenuated variants support the
hypothesis that preservation of structural and functional integrity of LSECs may be one determining factor in the severity of hepatitis.

In this study, we report that robust infection of LSECs by the highly virulent MHV3, in contrast to the attenuated 51.6- and YAC-MHV3 variants, promotes disturbances in their anti-inflammatory functions and secretion of vascular factors resulting in high release of inflammatory mediators and pro-coagulant Fgl-2 simultaneously with decrease in NO and IL-10 levels. Such MHV3-induced LSEC disorders correlated in vivo with higher hepatic inflammation, damages and viral replication as well as disturbances in leucocytes recruitment in mice infected by MHV3. We provide evidence that higher infection and proinflammatory activation of LSECs by MHV3 was related to a specific viral induction of TLR2 signalling. The aggravating role for TLR2 in hepatic inflammation and LSEC disorders was confirmed in MHV3-infected TLR2 KO mice in which hepatic damages, pro-vs anti-inflammatory cytokines ratio and LSEC-derived IL-10 production were significantly improved.

MATERIALS AND METHODS

Mice:
Female C57BL/6 (Charles River, St-Constant, Qc, Canada) and TLR2 knock out (KO) (C57BL/6-129 Tlr<sup>tm/Kir</sup>/J, Jackson Lab., Bar Harbour, MA) mice were housed in a HEPA-filtered air environment. All experiments were conducted with mice between 8 to 10 weeks
of age in compliance with the regulations of the Animal Committee of the University of Quebec in Montreal (CIPA).

Viruses

MHV3 is a cloned pathogenic substrain isolated from the liver of infected DBA2 mice. The MHV3 virus induces a rapid mortality in C57BL/6 mice within 3 to 4 days post infection (p.i.) (23). The escape mutant 51.6-MHV3 was selected from the pathogenic MHV3 virus cultured into L2 cells in the presence of S protein-specific A51 monoclonal antibodies (24). This variant induces a delayed mortality (5 to 9 days p.i.) and expresses low tropism for LSECs but retains ability to infect Kupffer cells (KC) (24). The non-pathogenic YAC-MHV3 variant is a cloned substrain produced in persistently infected YAC-1 cells, showing lower ability to replicate in LSECs and macrophages. Compared to the attenuated 51.6-MHV3 strain, this variant causes no mortality and induces efficient recruitment of innate immune cells allowing viral clearance from the liver within two weeks p.i. (31). All viruses were passaged less than three times onto L2 cells and their pathogenic properties were assessed routinely.

Isolation and purification of LSECs

Mice were euthanized and the portal vein was isolated and injected with 3 mL of HBSS 10mM HEPES followed by 3mL of digestion buffer consisting of 0.2% (w/v) collagenase A in HBSS 10mM HEPES (Sigma Aldrich, St-Louis, MO). The liver was then excised, injected several times with digestion buffer and dissociated by a 30 min incubation in 10 ml of
digestion buffer at 37°C on a shaking plate (200 RPM). The resulting cell suspension was passed through a sterile 70µM and a 40µM nylon mesh filter successively (Falcon, BD Biosciences, Mississauga, Ont., Canada) and centrifuged at 400g for 10 min. Cell pellet was resuspended in 3ml of RPMI 1640, layered at the top of a discontinuous 50%/25% Percoll gradient (Sigma Aldrich) and centrifuged at 800g for 20 min without brakes. The interphase between the two density cushions, containing enriched non-parenchymal cells, was collected and washed with PBS. LSEC were then purified using the positive selection PE kit (Stemcell, Vancouver, Canada) with an anti-CD146 monoclonal antibody, a specific marker of endothelial cells in liver (33), according to the manufacturer’s procedure. LSEC purity was analyzed by cytometry before each experiment and reached over 90%.

**In vivo viral infections**

Groups of 6-7 wild type C57BL/6 or TLR2 KO mice were intraperitoneally infected with $10^3$ TCID50 of MHV3, 51.6-MHV3 and/or YAC-MHV3. Mock-infected mice received a similar volume of PBS (Wysent). Mice were sacrificed by CO2 anoxia at 24, 48 and/or 72 h postinfection (p.i.) according to experiment. Liver and blood were collected and frozen for further analyses.

**Histopathological, transaminase levels and immunohistochemical analyses**

The histopathological analysis of liver was done by hematoxylin-eosin-safranine staining. Determination of serum ALT and AST transaminases was performed according to the IFCC primary reference procedures using Olympus AU2700 Autoanalyser® (Olympus Optical,
Tokyo, Japan). Immunolocalization of IL-33 and CAV-1 was performed on liver sections fixed in paraformaldehyde and embedded in paraffin incubated with primary goat anti-mouse IL-33 (R&D System Inc., Minneapolis, MN) or rabbit anti-mouse-CAV-1 (LSBio, Seattle, WA) for 1h in Ventana automated machine (Ventana Medical Systems, Inc. Tucson, AZ) and secondary HRP-conjugated rabbit anti-goat antibody (Dako, Markham, ON, Canada) or OmniMap anti-Rabbit-HRP (RUO) for 16 min. Double immunofluorescence stainings of IL-10 or IL-33 and CAV-1 were conducted on liver cryosections fixed in paraformaldehyde and incubated overnight with primary goat anti-mouse IL-10 (R&D System Inc., Minneapolis, MN) or primary goat anti-mouse IL-33 (R&D System) and rabbit anti-mouse-CAV-1 (LSBio) and then with DyLight-649-Anti goat Cy3-Anti Rabbit (Jackson ImmunoResearch, West Grove, PA) secondary antibodies and Hoechst counterstain (Invitrogen, Ontario, Canada). Slides were mounted (mounting medium, Invitrogen, Ontario, Canada), imaged with a Nikon’s Eclipse Ni-E Z1 microscope and analyzed using SpotAdvance software.

**Virus titration**

Frozen liver samples from 24 and/or 72 h MHV-infected mice were weighted and homogenized in cold PBS. Suspension was then centrifuged at 13000 RPM for 30 min and analyzed for viral detection. Viral titration was also performed on LSEC culture supernatants. Liver suspension and cell culture supernatants were 10-fold serial-diluted and tested for viral presence on L2 cells cultured in 96-well plates. Cytopathic effects (CPE), characterized by occurrence of large syncytia and cell lysis, were recorded at 72 h p.i. and
virus titers were determined according to Reed-Muench method and expressed as log_{10} tissue culture infectious dose (TCID)_{50}.

In vitro viral infections

Freshly isolated LSEC were seeded in 24-well plates at a density of 7.5 x 10^5 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics (Wyssent, St-Bruno, Qc, Canada). Cells were then infected with 0.1 multiplicity of infection (MOI) of infectious MHV3, YAC-MHV3 or 51.6-MHV3 and incubated at 37°C, under 5% CO2 for 24 to 72 h according to experiment. Cell culture supernatants were collected for ELISA assays and total RNA was extracted for qRT-PCR analysis.

siRNA transfection

LSECs were seeded in 24-well plates at 60,000 cells/ml and transfected with 25 nM of siRNA Flexitude premix (Qiagen, Cambridge, MA) targeting TLR2 mRNA (target sequence: CTCGTTTCTCCAGCATTTAAA) and with the AllStars Negative Control siRNA as nonsilencing transfection control for 36 h prior to infection for 24 h.

RNA isolation and RT-qPCR

RNA from in vitro infected LSEC was extracted using NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer procedure. Total RNA from frozen liver samples was extracted using TRIzol reagent (InVitrogen, Burlington, ON, Canada) and residual genomic DNA was removed with the Turbo DNA-free kit (Ambion, Austin, TX).
One μg of RNA was retro-transcribed into cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real time PCR amplification was carried out on 25ng cDNA using the HotStart-IT™ SYBR® Green qPCR Master Mix (USB Corporation, Cleveland, OH) on an ABI 7300 system (Applied Biosystems). Primer sets used are listed in Table I. Threshold cycle values (Ct) were collected and used for “ΔΔCt” analysis. The relative gene expression was normalized to HPRT as endogenous control and expressed as a ratio to gene expression in mock-infected mice livers or control (uninfected) LSECs in in vitro experiments (level arbitrarily taken as 1). The specificity of the PCR products was confirmed by melting curve analyses and all qPCR assays were run in duplicate.

**ELISA and nitric oxide assays**

Frozen liver samples were weighted and homogenized in NP40 lysis buffer (InVitrogen) completed with a protease inhibitor cocktail (Sigma Aldrich, St-Louis, MA) and 1 mM PMSF for protein extraction. Liver suspension was kept on ice for 30 min and centrifuged 10 min at 13000 RPM. Determination of IL-6, TNF-α (BD BioSciences, San Jose, CA) CXCL10, CCL2 (eBiosciences, San Diego, CA), CXCL1 and IL-33 (R&D Systems, Minneapolis, MN) in liver lysates and/or LSEC culture supernatants was carried out by ELISA tests. Levels of nitric oxide (NO) were quantified using the Griess reagent assay (InVitrogen) according to the manufacturer’s procedure.
Cytofluorometric studies

Livers were perfused with PBS through the portal vein to remove blood cell contamination prior to dissection. Liver tissues were then homogenized and hepatocytes were removed by sedimentation. Inflammatory cells were enriched using 35% Percoll gradient (Sigma Aldrich) and red blood cells were lysed with a Tris-buffered ammonium chloride solution. A million (10⁶) of leucocytes were incubated with anti-CD16/32 antibodies (BD Biosciences) to block non-specific binding. Cells were incubated with optimal dilutions of anti-CD3-V500, anti-Gr1-V450, anti-CD11b-PE-Cy7, anti-CD19-APC, anti-CD4-FITC, anti-NK1.1-PerCP-Cy-5.5 and anti-CD8-APC-Cy7 antibodies (BD Biosciences) and fixed in PBS containing 2% FCS, 0.01 M sodium azide and 2% formaldehyde. Stained cells were analyzed on a FACS Aria II® flow cytometer using BD FACS Diva software (BD Bioscience) and the data were processed using CXP software (Beckman Coulter, Mississauga, ON, Canada). Dead cells and doublet cells were excluded on the basis of forward and side scatter and analyses were performed on 10,000 events recorded. Myeloid cells, gated by high side scatter, were assessed for CD11b and Gr1 to enumerate macrophages (CD11b+Gr1inter) and neutrophils (CD11b+Gr1high). Lymphoid cells were gated according to FSC/SCC and first assessed for NK1.1 and CD3 expression to discriminate NK from NKT cells. CD3+NK1.1- T cells were further gated to allow determination of CD4+ and CD8+ subpopulations. B lymphocytes were determined by CD19+CD3- expression.
Statistical analyses

Data are expressed as means ± the standard error of the mean. Statistical analyses for in vitro studies were performed with Student’s t-test comparing uninfected (control) to virus-infected cells or virulent to attenuated MHV3 infections. Multiple group analyses were conducted for in vivo studies and data obtained by qPCR, ELISA and viral titration were evaluated by one-way ANOVA test with posthoc Tukey test using PASW Statistics software (PASW version 18, IBM SPSS Inc. Chicago, IL). Values of p ≤ 0.05 were considered as significant.

RESULTS

Lower tropism of attenuated MHV3 variants for LSECs is associated with less severe damages and viral replication in the liver

Previous studies have shown that acute infections by the attenuated 51.6- and YAC-MHV3 variants resulted in milder or subclinical hepatitis respectively in comparison to fulminant hepatitis induced by the parental virulent MHV3 strain (24, 31). We first aimed to compare the evolution of damages, inflammatory infiltrates and viral replication in the liver of mice infected by virulent and attenuated MHV3 strains. C57BL/6 mice were i.p. infected with either MHV3 or attenuated viruses for 24 to 72 h p.i., and blood and livers were collected for clinical, histopathology and viral titer analyses. Liver histopathology from virulent MHV3-infected mice showed inflammatory foci surrounding necrotic cells at 24 h and 48 h p.i. which disappeared at 72 h p.i. while hepatocyte necrosis became extensive (Fig. 1A and B).
Infection with 51.6-MHV3 revealed delayed occurrence of inflammatory foci at 48 h p.i. with barely detectable hepatic damages while YAC-MHV3 induced few small inflammatory infiltrates with no observable hepatic necrosis areas even at 72 h p.i. (Fig. 1 A and B). Extensive hepatic damages in virulent MHV3-infected mice correlated with high levels of blood ALT and AST transaminases at 72 h p.i. (p ≤ 0.001) (Fig. 1 C and D) and sooner and higher viral replication than in attenuated 51.6-and YAC-MHV3 variants-infected mice (p ≤ 0.01 to 0.001) (Fig. 1 E and F).

Attenuated MHV3 strains induce lower Fgl-2, CAV-1 and IL-33 expression in the liver than virulent MHV3

Vascular and structural disorders in LSECs were reported in viral hepatitis, correlating with hepatic damages (19, 27, 29). Indeed, previous studies have demonstrated that induction of Fgl-2, a prothrombinase expressed by LSECs promoting vascular thrombosis and hepatic inflammation, correlated with MHV3-induced fulminant hepatitis (28). Moreover, a direct association between capillarization (lack of fenestrations) of LSECs in livers from HCV-infected patients and an overexpression of caveolin-1 (CAV-1), a key component of LSECs fenestrations, was recently evidenced (34). To verify whether the lower severity of hepatitis induced by the attenuated MHV3 strains was associated with lower dysfunctions in LSECs, mRNA levels for CAV-1 and Fgl2 were quantified by qRT-PCR in livers from all infected groups of mice. Intrahepatic expression of CAV-1 was also localized by immunohistochemistry staining in MHV3- and 51.6-MHV3-infected mice. As shown in
figure 2A, higher increase of CAV-1 mRNA levels were observed at 48 h p.i. in the liver of MHV3-infected mice while lower or no induction was noted in 51.6- and YAC-MHV3-infected mice, respectively (p ≤ 0.05 and 0.001). Immunolocalization of CAV-1 revealed specific expression in LSECs and confirmed higher induction in the liver of MHV3- than 51.6-MHV3-infected mice (see black arrow, Fig. 2B). Gene expression of Fgl2 increased as soon as 24 h p.i. in mice infected with MHV3 while it was delayed and lower in 51.6-MHV3 infection or not induced in YAC-MHV3-infected mice (p ≤ 0.05 to 0.001) (Fig. 2C). In addition, Fgl-2 mRNA reached higher levels in the liver of virulent than attenuated MHV3-infected mice (p ≤ 0.001).

We have recently reported that MHV3 infection was associated with early release of IL-33, an alarmin mainly secreted by injured LSECs (29). We aimed to verify whether lower tropism of attenuated MHV3 strains for LSECs may be associated with lower expression of IL-33. To test this hypothesis, mRNA expression, production and localization of IL-33 were assessed by qRT-PCR, ELISA and IHC, respectively, in livers from MHV3 and 51.6-MHV3 infected groups of mice. As shown in figures 2D and E, gene expression and release of IL-33 increased only in the liver of MHV3-infected mice (p ≤ 0.001) while it was rather not induced or inhibited in mice infected with 51.6- or YAC-MHV3 (p ≤ 0.05 to 0.01). IHC stainings indicate that expression of IL-33 was only induced in the liver of MHV3-infected mice and mostly localized in LSECs, and at a lesser extent in hepatocytes nuclei (Fig. 2F). Induction of IL-33 in LSECs was confirmed by a double immunostaining IL-33/caveolin-1 in livers from MHV3-infected mice at 48 h p.i. and to a lesser extent at 72 h p.i. (Fig. 2G).
Virulent MHV3 infection leads to an imbalance of pro- over anti-inflammatory mediators in the liver in contrast to infection by attenuated MHV3 strains. Given the crucial role of LSECs in the control of liver inflammation through production of anti-inflammatory cytokines, we presumed that dysfunctions of LSECs in MHV3-infected mice may favor the induction of a pro-inflammatory state in the liver. To verify this hypothesis, levels of anti-inflammatory cytokines (IL-10, TGF-β) and proinflammatory cytokines (IL-6, TNF-α) and chemokines (CCL2, CXCL1, CXCL10) were assayed by qRT-PCR (from 24 to 72 h p.i.) and ELISA (72 h p.i.) in the liver of all groups of infected mice. As indicated in the figure 3 sect.I A and B, mRNA expression levels and production of IL-10 were markedly increased in the liver of 51.6-MHV3- and YAC-MHV3-infected mice when compared to lower levels induced in MHV3-infected mice (p ≤ 0.05 to 0.001). At a lesser extent, TGF-β mRNA and production levels were also higher induced in the liver of 51.6-MHV3-infected mice, especially at 24 and 48 h p.i. (p ≤ 0.05 to 0.001)(Fig. 3 C-D). To verify whether IL-10 induction in the liver of 51.6-MHV3-infected mice occurred in endothelial cells (EC), immunohistochemistry stainings using specific antibodies to IL-10 and CAV-1 (EC marker) were conducted on liver sections. In comparison to staining in mock-infected mice, IL-10 expression in livers from 51.6-MHV3-infected mice was induced in the parenchyma and in venous and sinusoidal ECs whereas occurrence and intensity of IL-10 staining were weaker in MHV3-infected mice (Fig.3 sect. II).
On the other hand, mRNA expression and production levels of intrahepatic IL-6 and TNF-alpha were higher up-regulated in MHV3- than 51.6- or YAC-MHV3-infected mice (p ≤ 0.05 to 0.001) (Fig. 3 sect.I E-H). In the same line, transcription and production levels of the chemokines CXCL1, CCL2 and CXCL10 increased throughout infection by MHV3 but were delayed or dramatically reduced in 51.6- or YAC-MHV3-infected mice (p ≤ 0.05 to 0.001) (Fig. 3 sect.III A-F).

**Virulent MHV3 induces higher expression of TLRs and helicases in the liver than attenuated MHV3 strains**

Induction of inflammatory response during viral infection is triggered upon activation of PRRs, such as TLRs and helicases, by viral products. Several studies have reported increased TLR expression in viral hepatitis, correlating with liver inflammation (reviewed in 17). We explored the hypothesis that higher release of inflammatory mediators in MHV3 infection may be related to increased expression of TLRs or helicases in the liver. Thus, the kinetics of surface TLR-2 and -4, endosomal TLR-3 and -7, and helicase RIG-1 and MDA-5 gene expression were compared by qRT-PCR in the liver of infected mice from 24 to 72 h p.i. As shown in figure 4A, TLR2 expression steadily higher increased over the course of infection with MHV3 while its induction was drastically reduced in mice infected with the attenuated variants (p ≤ 0.01 to 0.001). Expression levels of TLR3, TLR4, RIG-1 or MDA-5 genes were only or more increased during MHV3 than in 51.6- or YAC-MHV3 infections.
albeit markedly lesser than TLR2 (p ≤ 0.05 to 0.001) (Fig. 4B, C, E and F), whereas levels of TLR7 were unaffected by neither viruses (Fig. 4D).

These data suggest that higher levels of inflammatory mediators in the liver of MHV3-infected mice may be associated with preferential and higher induction of PRRs, especially TLR2, by the virulent MHV3.

Hepatic proinflammatory state in virulent MHV3-infected mice leads to rapid but transient intrahepatic recruitment of inflammatory cells and decrease of B, CD4 and CD8 lymphocyte subsets

LSECs are responsible for the recruitment and transmigration of leucocytes during liver inflammation (35). We postulated that higher production of chemokines in the liver of MHV3-infected mice induced higher recruitment of inflammatory cells than in mice infected with attenuated virus strains. Such hypothesis was supported by higher occurrence of inflammatory infiltrates in the liver of MHV3-infected mice at 24h p.i. (see Fig. 1A). To determine leukocyte subsets recruited into the liver, intrahepatic mononuclear cells were isolated at 24 and 48 h p.i. from all groups of mice, immunolabeled, and the percentages of NK-T (NK1.1+CD3+) and NK (NK1.1+CD3-) cells, neutrophils (CD11b<sup>hi</sup>Gr1<sup>hi</sup>), macrophages (CD11b<sup>hi</sup>Gr1<sup>int</sup>), B (CD19+) and T (CD8+ and CD4+) cells were analyzed by cytometry and compared to cells from mock-infected mice. As shown in figure 5, section I-A, percentages of NK-T cells transiently decreased in the liver of MHV3-infected mice (p ≤
0.001) differing to that seen livers from 51.6- and YAC-MHV3 infected mice (p ≤ 0.05 to 0.01). NK cell percentages higher increased in MHV3- than 51.6-MHV3-infected mice while it decreased in YAC-MHV3 (p ≤ 0.05 to 0.001) (Fig. 5, section I-B). Neutrophils, however, were earlier and higher recruited into the liver of MHV3- than in 51.6- and YAC-MHV3-infected mice (p ≤ 0.05 and 0.001) (Fig. 5, section I-C). Percentages of intrahepatic macrophages more increased in MHV3-infected mice (p ≤ 0.05 to 0.001) (Fig. 5, section I-D). Regarding lymphocyte subsets, B and CD4+ cell percentages stronger decreased in the liver of MHV3-infected mice (p ≤ 0.05 and 0.001) (Fig. 5, section I-E-F) while CD8+ cells were higher reduced by 51.6- or YAC-MHV3 infections (p ≤ 0.05 to 0.001) (Fig. 5, section I-G).

Since a substantial decrease in total number of isolated intrahepatic cells was noted over infection time with MHV3 only, the analysis of absolute numbers of each cell subset rather than the relative percentages better reflects the recruitment of inflammatory cells. Cell numbers were then determined, using the percentage of each subset reported to total number of isolated cells in the liver of each mice. As shown in figures 5, section II-A and B, NK-T cells decreased only in the liver of MHV3-infected mice (p ≤ 0.01 and 0.001) while total NK cells were not altered in all infected groups. Number of neutrophils, however, earlier but transiently increased at 24 h p.i. in MHV3-infected mice while they were delayed or lower recruited in livers from 51.6- and YAC-MHV3-infected mice respectively when compared to MHV3 infection (p ≤ 0.05 to 0.001) (Fig. 5, section II-C). In contrast to that observed with percentages, numbers of intrahepatic macrophages increased in the liver of mice
infected with attenuated YAC- and 51.6-MHV3 strains (p ≤ 0.01 and 0.001) but not with MHV3, but such increases were not statistically significant when compared to MHV3 infection (Fig. 5, section II-D). The numbers of B and T (CD4 and CD8) cells were also dramatically impaired over the course of infection by MHV3, but were less or not altered by 51.6- or YAC-MHV3 infections or transiently increased at 24 h p.i. in YAC-MHV3-infected mice (p ≤ 0.05 to 0.001) (Fig. 5, section II-E and G).

Permissivity of LSECs to MHV3 strains correlates with virulence.

We next attempted to characterize the effect of virulent and attenuated MHV3 infection on functional and structural integrity of LSECs in vitro. LSECs were isolated from the liver of C57BL/6 mice and purified by Percoll gradient followed by immunomagnetism using the anti-CD146 antibodies. As shown in the figure 6A, 87 to 91% of isolated cells expressed the endothelial markers CD146, CD54 (ICAM-1), and CD31 (PECAM-1) but not the macrophage marker F4/80. Isolated LSECs were then infected by the MHV3 strains and viral replication as well as CPE were monitored from 24 to 120 h p.i. CPE in LSECs was characterized by cell lysis and rounded shaped cells instead of typical MHV-induced giant syncytial cells (usually observed in L2 cells) and occurred sooner in virulent MHV3-infected culture as cells were totally lysed by 72 h p.i. In contrast, CPE in cells infected by attenuated strains were delayed to 72 h p.i. and increased up to 120 h p.i. (Fig. 6B) (p≤0.001 when compared with MHV3-infected cells). Infectious viruses in supernatants from MHV3-infected LSECs were detected at 48 h p.i and then started to decrease as cell damages
became extensive whereas titers of 51.6- and YAC-MHV3 were only detected after 96 or 120 h p.i. (Fig. 6C) (p≤0.001 when compared with MHV3-infected cells).

Virulent but not attenuated MHV3 strain induces Fgl2, IL-33 and caveolin-1 expression and alters NO production by LSECs

To confirm that attenuated MHV3 variants, in contrast to MHV3 virus, do not disturb LSECs integrity and vascular factors, as observed in vivo, expression level of the alarmin IL-33 and the prothrombinase Fgl-2 were evaluated respectively in infected LSECs. As expected and shown in the figures 7A to C, gene expression and release of IL-33 increased throughout infection only in MHV3-infected cells (p≤0.05 to 0.001) while Fgl2 expression was up-regulated at 48 h p.i. in MHV3-infected cells only (p≤0.05 to 0.001). Lower levels of Fgl2 mRNA at 72 h p.i. (p≤0.05) reflected total cell lysis in MHV3-infected LSECs.

MHV3 replication was already shown to be controlled in vitro by nitric oxide (NO) (36). Since LSECs constitutively release NO, a vasodilator factor regulating sinusoidal blood flow (37), we verified whether higher replication of MHV3 in LSECs may result from defect in NO production by quantifying NO levels in culture supernatants. As shown in figure 7D, release of NO was reduced only in MHV3-infected cells differing thus with 51.6-MHV3- or YAC-MHV3-infected cells (p≤0.01) (Fig. 7B). Since NO production was reported to be negatively regulated by CAV-1 through inhibition of endothelial nitric oxide synthase (eNOS) activity (38), we investigated whether NO alteration by MHV3 infection was
associated with up-regulation of CAV-1 expression in infected LSECs, as seen in the liver of MHV3-infected mice. In agreement with our in vivo observations, mRNA expression level of CAV-1 increased only in MHV3-infected LSECs at 24 and 48 h p.i. (fig. 7E) (p≤0.05 to 0.001).

Virulent MHV3, in contrast to attenuated strains, promotes LSECs conversion into a proinflammatory profile

MHV3-infected mice exhibited higher inflammatory response in the liver than mice infected by attenuated strains, suggesting defect in the control of inflammation by LSECs. LSECs were already reported to produce IL-6 upon infection by MCMV (5), indicating a possible switch from an anti- to pro-inflammatory phenotype once infected. We thus speculated that LSECs infected by MHV3, in comparison to attenuated strains, may adopt a preponderant proinflammatory profile. To address this, mRNA expression and production levels of anti-inflammatory (IL-10 and TGF-β) and proinflammatory (IL-6, TNF-α) cytokines produced by infected LSECs were determined by qRT-PCR and ELISA assays. As shown in Fig. 8 section I-A and B, mRNA expression and production of IL-10 slightly increased at 24 h p.i. in MHV3-infected LSECs but decreased thereafter below the basal level in uninfected cells (p< 0.05 and 0.001). Consistent with the up-regulation of IL-10 in the liver of mice infected with attenuated MHV3 strains, IL-10 levels rapidly or progressively higher increased in 51.6-MHV3- and YAC-MHV3- than in MHV3-infected LSECs up to 72 h p.i. (p≤0.05 to 0.001). TGF-β expression, however, was not or slightly induced in cells infected by the
attenuated strains (p≤0.05) but was less inhibited in attenuated virally-infected cells than in virulent MHV3-infected cells (p≤0.05 and 0.01) (Fig. 8 section I-C and D). These results suggest that MHV3 infection suppresses anti-inflammatory function of LSECs whereas attenuated strains rather promote it.

TNF-α mRNA levels, however, rather transiently increased only in MHV3-infected LSECs at 24 h p.i. (p≤ 0.01) (Fig. 3 section. I-E) and were completely inhibited at 72 h p.i. by all MHV3 strains (p≤ 0.01). Amounts of TNF-α released in supernatants of infected LSECs, however, increased in all infected cells (p≤ 0.001) but remained higher in MHV3 and YAC-MHV3- than 51.6-MHV3-infected cells (p≤ 0.05) (Fig. 8 section I-F). The mRNA expression of IL-6 reached higher levels in cells infected by virulent MHV3 than attenuated strains at 24 h p.i. only (p≤ 0.05 to 0.001) (Fig. 8 section I-G), correlating with higher release in supernatant of MHV3-infected cells (p≤ 0.01) (Fig. 8 section I-H).

LSECs were also reported to secrete chemokines upon infection by Dengue virus and to enhance their production in chronic inflammatory liver disease (39, 40). Thus, we presumed that MHV3-infected LSECs may produce higher levels of chemokines. As shown in figure 3, section II-A to D, CXCL1 and CCL2 expressions were higher upregulated in MHV3-infected LSECs than in 51.6- and YAC-MHV3-infected cells at 24 and 48 h p.i. leading to higher amounts released in cell supernatants (p≤ 0.05 to 0.001). CXCL10 gene expression and production levels only increased in MHV3-infected LSECs (p≤ 0.05 to 0.001) (Fig. 8 section II- E and F).
Proinflammatory activation of LSECs by MHV3 depends on TLR2 signalling

We have previously shown that induction of inflammatory cytokines by MHV3 in *in vitro* infected macrophages depended on TLR2 signaling (37). In addition, Liu et al. (9) recently demonstrated that TLR2 activation on LSECs reversed their anti-inflammatory functions. Since TLR2 was strongly up-regulated in livers from MHV3-infected mice, we aimed to investigate whether TLR2 was involved in the conversion of LSEC towards a proinflammatory profile. We first sought to determine whether MHV3 increased TLR2 expression on LSECs. As shown in figure 9A, levels of TLR2 mRNAs were significantly higher in cells infected by MHV3 than attenuated strains at 24 h p.i. (p≤ 0.05 to 0.01). To address whether TLR2 was involved in cytokine response and viral replication in infected LSECs, TLR2 expression was abrogated by siRNAs prior to infection and IL-6 and CXCL1 mRNA levels were determined by qRT-PCR at 24h p.i. Viral replication of MHV3, but not 51.6 and YAC-MHV3, was significantly reduced following TLR2 knockdown (p≤ 0.001) (Fig. 9B). A markedly decreased expression of IL-6 and CXCL1 and an up-regulation of IL-10 levels were also observed in MHV3-infected cells rendered defective for TLR2 while no difference was noted in cells infected with the attenuated variants (p≤ 0.001)(Fig. 9C to E) (p≤ 0.001). These results suggest that higher tropism and pro-inflammatory inducible capacities of MHV3 in LSECs reflect its unique ability to activate TLR2 signalling.
TLR2 exacerbates liver damage and increases viral replication in mice infected by virulent but not attenuated MHV3 strains.

We already reported that MHV3-induced acute hepatitis was less severe in TLR2 KO mice (41). To verify whether TLR2 is differentially involved in the evolution of hepatitis induced by virulent and attenuated MHV3 strains, groups of wild type (WT) C57BL/6 and TLR2 knock-out (KO) mice were i.p. infected with MHV3 or 51.6-MHV3. Survival rate was monitored and liver damage and viral load were evaluated at 72 h p.i. As shown in figure 10A and B, survival of TLR2 KO mice infected by MHV3, but not 51.6-MHV3, was prolonged when compared to respective infected WT mice (p ≤ 0.001). Accordingly, histopathological analysis of the liver revealed less and smaller necrotic foci in MHV3-infected TLR2 KO mice than in WT mice whereas comparable and barely detectable hepatic damages were noted in TLR2 KO and WT mice infected with 51.6-MHV3 (Fig. 10C).

In addition, viral replication of MHV3 at 72 h p.i. was lower in the liver of infected TLR2 KO than WT mice whereas 51.6-MHV3 replication was similar in both mice strains (Fig. 10D) (p ≤ 0.001). Taken together, these results suggest that TLR2 aggravates hepatic damages and viral replication in mice infected by virulent but not attenuated MHV3 strains.

TLR2 activation by virulent MHV3 decreases IL-10 and increases inflammatory cytokines and chemokines expression.
It was previously reported that hepatic levels of IL-6 and TNF-α were reduced in MHV3-infected TLR2 KO in comparison to C57BL/6 mice, suggesting a role for TLR2 in MHV3-induced release of inflammatory factors (41). Thus, we speculated that MHV3, in contrast to 51.6-MHV3, may promote a pro-inflammatory cytokine profile in the liver through TLR2 activation, such as observed in *in vitro* infected LSECs. To test this hypothesis, expression levels of several inflammatory and anti-inflammatory factors were compared between livers from TLR2 KO and wild type (WT) mice infected with both viruses. As shown in Table II, lower mRNA expression of TNF-α, IL-6, CXCL1, CCL2, CXCL10 and higher IL-10 levels occurred in the liver of MHV3-infected TLR2 KO mice compared to WT mice (p ≤ 0.001) whereas levels of Fgl2 and IL-33 were similar in both mouse strains. In contrast, no difference was observed between cytokine profile in 51.6-MHV3-infected WT and TLR2 KO mice, albeit a slight reduction of CXCL10 expression was noted in TLR2 KO mice (p ≤ 0.05). Given the importance of IL-10 in the control of hepatic inflammation, we aimed to determine whether higher levels in livers from MHV3-infected TLR2 KO mice reflected higher production by ECs. A double immunohistochemistry staining of IL-10 and CAV-1 on liver sections revealed that expression of IL-10 in ECs was effectively higher in livers from MHV3-infected TLR2 KO than WT mice (Fig. 11 compared with Fig. 3 section II).

**DISCUSSION**

In this work, we investigated the role of LSECs in hepatic inflammation during acute viral hepatitis process using the MHV3 model of infection. We demonstrated that the severity of
hepatitis, viral replication and hepatic inflammation correlated with permissivity of LSECs for MHV3 strains and subsequent structural and functional disturbances. We showed that in vitro infection of LSECs by the virulent MHV3, in contrast to the attenuated 51.6- and YAC-MHV3 variants, resulted in earlier cell damage and disorders in inflammatory and vascular factors, as reflected by high release of inflammatory cytokines/chemokines and pro-coagulant Fgl-2 and a decrease in NO and IL-10 levels. We evidenced that the higher replication rate and proinflammatory activity of MHV3 in LSECs was associated with its specific activation of TLR2 signalling in LSECs and exposed that TLR2 is a key factor of hepatic inflammation and LSEC-derived IL-10 disorders in MHV3-induced fulminant hepatitis.

LSECs, lining the hepatic sinusoids, mediate liver tolerance under physiological conditions (reviewed in 1) but these cells are target of many hepatotropic viruses. The consequence of LSECs infection in inflammatory liver diseases such as viral hepatitis has never been investigated. We have shown that MHV3 infection induced differential structural and functional disorders in LSECs according to strain virulence. Indeed, the highly virulent MHV3 replicated faster and higher in LSECs leading to occurrence of CPE such as change in morphology (rounded cells) and cell lysis from 48 h p.i. Previous reports have already shown that in vivo and in vitro infections of LSECs by MHV3 were associated with cell damages and loss of fenestrations (27), but no syncytial cells were observed. However, MHV3 did not replicate in LSECs as fast as it usually does in in vitro cultured cells since no viral burden was detected until 48 h p.i. while MHV3 titers are detectable within 24 h p.i. in
Our results are nevertheless in accordance with those from Pereira et al. (26) who have shown that MHV3 replicates more rapidly in Kupffer cells (KC cells) than in LSECs in vitro, suggesting that LSECs may transiently control the viral replication. In agreement, replication of the attenuated 51.6- and YAC-MHV3 variants was delayed to 96 or 120 h p.i. and was associated with barely detectable CPE, reflecting their weaker tropism for LSECs. It was recently reported that LSECs exhibit high clearance capacity of circulating viruses (42, 43), suggesting that they may express high ability to sequester attenuated but not virulent MHV3 particles. However, as replication of MHV3 variants in LSECs was delayed but not aborted rather suggests a host cell-dependent control mechanism of viral replication. Indeed, preliminary results showed higher antiviral IFN-β response in LSECs infected by attenuated MHV3 strains (results not shown). The low IFN-β response in virulent MHV3-infected LSECs may be related to specific viral evasion mechanisms from host viral sensors or interference with downstream signaling pathways. We have observed that MHV3, in contrast to attenuated strains, neither induced TLR3 nor RIG-I expression in LSECs (results not shown), suggesting lower detection by these viral sensors. Further work should address whether viral products or evasion strategies are involved in MHV3-induced impairment of IFN-β response in LSECs.

The inability of attenuated MHV3 variants to establish a rapid infection in LSECs correlated with a less severe hepatitis. Indeed, 51.6-MHV3 infection resulted in lower viral replication, transaminase levels and liver damages than MHV3 infection. The 51.6-MHV3 variant only
differs from the pathogenic MHV3 by its weaker tropism for LSECs but retained its virulence for hepatocytes, KCs and Ito cells (24), suggesting that resistance of LSECs to viral replication may protect against fulminant hepatitis. Similarly, the non pathogenic YAC-MHV3, also expressing low ability to replicate in LSECs, induced light and transient hepatic lesions, reinforcing the importance of functional integrity of LSECs in the evolution of viral hepatitis. Indeed, less severe hepatic damages and viral load in mice infected with the attenuated variants may possibly result from a better early control of viral replication by LSECs leading to reduced transmission of viral progeny to the hepatic parenchyma. In agreement, a delayed replication of MHV3 in LSECs was suggested as a crucial step in the resistance of various strains of mice to MHV3 infection by allowing time for the local and systemic responses to clear the infective particles (26).

We report here for the first time that in vitro MHV3 infection promotes a proinflammatory activation of LSECs. Indeed, MHV3 induced higher levels of IL-6, TNF-α and chemokines in LSECs than attenuated strains and inhibited their basal release of IL-10 while attenuated strains rather enhanced it. These inflammatory disorders in LSECs correlated with higher ratios of intrahepatic inflammatory over anti-inflammatory mediators in the liver of MHV3-infected mice, suggesting that LSECs may have lost their ability to control inflammation. In agreement, IL-10 staining was significantly lower in ECs from the liver of mice infected by MHV3 than by attenuated strains. The importance of IL-10 production by LSECs in the suppression of pro-inflammatory cytokine release by Th1 and Th17 was recently evidenced by Carambia et al. (44). In addition, LSECs were recently shown to be more efficient than
KCs in tolerizing autoreactive Th1 cells via IL-10 (2). The lower inflammatory profiles in livers from 51.6- and YAC-MHV3-infected mice are in line with our previous observations (30). The highly attenuated YAC-MHV3 infection correlated with lower induction of inflammatory mediators than 51.6-MHV3 infection. Higher levels of anti-inflammatory IL-10 and immunosuppressive PGE2 were already reported in the liver of YAC-MHV3- than 51.6-MHV3-infected mice (30), suggesting that the highly attenuated phenotype of YAC-MHV3 may reflect the preservation of integrity of LSECs and other yet unidentified hepatic cells. Since YAC-MHV3, unlike 51.6-MHV3, was shown to lower replicate in macrophages in vitro (45), it is plausible that preservation of KCs tolerant functions may further contribute to lower inflammatory responses during YAC-MHV3 infection. Altogether, results from YAC-MHV3 and 51.6-MHV3 infections strenthen the importance of LSECs structural and functional integrity in restricting hepatic inflammatory response and subsequent damage. In agreement, activation of LSECs towards a pro-inflammatory profile was pointed out as a critical component of intrahepatic inflammation in hepatic fibrosis (40).

Differences in LSECs cytokine profile according to infection by pathogenic or attenuated MHV3 strains may reflect differential PRRs induction and activation by viral fixation and/or replication. We have already demonstrated that IL-6 and TNF-α production by MHV3-infected macrophages resulted from TLR2 activation by the surface (S) viral protein (41). The production of TNF-α by LSECs is known to depend on TLR1 to 4, -6 and -8 while IL-6 is produced following activation of TLR1 to 4 only (4, 17). It has been recently demonstrated that TLR1/2 ligand (PamC3), but not TLR3 ligand (poly I:C) or LPS, reverted...
the suppressive properties of LSECs (9). We have shown that virulent MHV3 strain highly
induced TLR2 expression on cultured LSECs and that TLR2 knockdown abrogated IL-6 and
CXCL1 induction only in LSECs infected by MHV3. Indeed, the proinflammatory activity
of MHV3 may be related to its unique ability to induce TLR2 signalling. In agreement, lower
levels of inflammatory cytokines and chemokines were observed in the liver of MHV3-
infected TLR2 KO mice, correlating with milder hepatic damages and delayed mortality of
mice. Thus, TLR2 activation may represent one determining and differential factor involved
in the severity of virulent vs attenuated MHV3-induced hepatitis. In line with this
hypothesis, survival rate, inflammatory response and liver damage were similar in TLR2 KO
and WT mice infected by 51.6-MHV3 and were comparable to that observed in MHV3-
infected TLR2 KO mice. Furthermore, IL-10 levels were significantly higher in MHV3-
infected TLR2 KO mice, with increased expression on ECs and also on some CAV-1
negative cells, suggesting that specific activation of TLR2 by the virus could be one
mechanism by which MHV3 reverts the anti-inflammatory phenotype, at least, in LSECs.
Supporting this assumption, IL-10 expression was significantly up-regulated in in vitro
MHV3-infected LSECs treated with siTLR2, suggesting an inhibitory role for TLR2 on IL-
10 induction by MHV3. Accordingly, TLR2 activation has already been shown to
temporarily reverse Tregs suppressive functions (46, 47). Further work will be done to
identify the other IL-10-producing CAV-1-negative cells during the MHV3 infection.

TLR2 may also potentiate MHV3 infection as viral replication was significantly reduced in
the liver of TLR2 KO mice and in cultured LSECs rendered defective for TLR2. In addition,
activation of TLR2 by MHV3 may additionally account for its higher replication rate in LSECs since the replication of 51.6-MHV3 was not influenced by TLR2 in infected LSECs or mice. It has been demonstrated that MHV replication depends on the activation of the P38 MAPK at the beginning of the replicative cycle (48). Thus, it is conceivable that activation of TLR2 by MHV3 on LSECs optimizes P38 MAPK activation, predisposing to more efficient viral replication. Since TLR2 is also but less expressed by other resident or recruited cells in the liver, such as KCs, neutrophils and hepatocytes, as observed in preliminary experiments, we can hypothetize that several TLR2+ cells permissive to MHV3 infection may act synergistically in promoting viral replication and hepatic inflammation. Indeed, preliminary in vitro results revealed that production of inflammatory mediators and viral replication in MHV3-infected hepatocytes and macrophages was enhanced by TLR2. Further work is in progress to clarify the mechanistic implication of TLR2 in MHV3 replication and the role of recruited and resident TLR2+ inflammatory cells in hepatic inflammation and damage.

The differences in chemokine levels induced by the pathogenic and attenuated MHV3 strains may explain the differences in recruited intrahepatic leukocyte subsets. Indeed, lower levels of CXCL1 and CCL2 in livers from 51.6- and YAC-MHV3-infected mice correlated with delayed or lower intrahepatic recruitment of neutrophils and macrophages explaining thus the smaller inflammatory foci without extensive necrosis areas seen in livers from these mice. Unexpectedly, neutrophils were only transiently recruited and numbers of NK-T, T and B lymphocytes progressively decreased throughout MHV3 infection despite high
induction of chemokines. We have previously demonstrated that intrahepatic NK an NK-T cells undergo higher apoptosis, and that B and T cells are stronger depleted in lymphoid organs during MHV3 than YAC-MHV3 (30, 31, 49), thus altering lymphocyte recruitment or turnover into the liver. The highly attenuated YAC-MHV3 infection, compared to MHV3, was also related with effective activation of CD8(+) cells (32).

In addition, impairment of intrahepatic leukocyte populations and severe liver injury in MHV3-infected mice may also be connected to disturbances in LSEC-derived vascular factors. We have demonstrated that MHV3, unlike attenuated variants, significantly altered NO release by LSECs. Susceptibility of mice to MHV3 infection has already been inversely correlated with NO levels in the liver, but the mechanism was not elucidated (50). The constitutive expression of NO by LSECs is essential for the regulation of intrahepatic sinusoidal blood flow and protects against liver diseases. Indeed, impairment of NO release by LSECs has been associated with hepatic microvascular dysfunction and portal hypertension in liver pathological conditions such as fibrosis and cirrhosis (37). In cirrhotic livers, NO defect has been linked to an overexpression of CAV-1 on LSECs, a negative regulator of the endothelial NO synthase activity (38). We have observed that reduced NO levels in LSECs correlated with a concomittent up-regulation of CAV-1, supporting that MHV3-induced NO impairment is indirectly related to CAV-1 induction. Furthermore, we have demonstrated that expression of the procoagulant Fgl2 increased only in LSECs infected by MHV3. MHV3-induced expression of Fgl2 has already been reported in endothelium of intrahepatic veins and sinusoids and was associated with severe intravascular
coagulation, ischemia and liver necrosis in MHV3-infected mice (28). In agreement, liver histopathological analysis revealed vascular thrombosis and fibrin deposition in hepatic veins and sinusoids from 48h p.i. in MHV3-infected mice only (results not shown). No difference in Fgl2 levels was observed between MHV3-infected WT and TLR2 KO mice, indicating that induction of Fgl2 in LSECs is TLR2-independent. Since Fgl2 expression in LSECs was reported to be promoted by the MHV nucleocapsid protein and TNF-α (51, 52), we can assume that higher induction of Fgl2 in the liver of MHV3-infected mice may reflect higher hepatic TNF-α levels and viral replication rate in LSECs. Thus, the combined effect of CAV-1/NO imbalance and Fgl2 induction during MHV3 infection may contribute to alter leukocyte recruitment and aggravate hepatitis in disturbing hepatic microcirculation.

The alarmin IL-33 was shown to be up-regulated in LSECs during chronic HBV and HCV infections and acute liver failure but the mechanism is elusive (21, 22). In agreement with our previous report, MHV3 infection increased IL-33 production in both LSECs and hepatocytes (29). Our results showed that IL-33 expression in LSECs was only increased by virulent MHV3 and was not modulated by TLR2, suggesting that IL-33 release is rather a consequence of MHV3-induced cell damages as necrotic cells in the liver were shown to secrete alarmins such as HMGB-1 and IL-33 (53). In addition, high IL-33 serum level was associated with liver damages in HBV and HCV infections, indicating that IL-33 could be considered as a predictive indicator of viral hepatitis evolution, as previously suggested (54, 55).
Using the MHV3 animal model of viral acute hepatitis, this work suggests a novel viral-promoted mechanism of hepatic inflammation and damages involving disorders in LSEC-derived inflammatory and vascular factors. The use of MHV3 variants expressing weak tropism for LSECs allowed us to better discriminate the importance of LSECs, over other hepatic cells, in tolerance/inflammation imbalance during acute viral infection. Our results support that induction of TLR2-dependent reversion of LSECs anti-inflammatory functions by MHV3 may participate in the pathological inflammatory response that predisposes to fulminant hepatitis. Unlike MHV3, HCV and HBV do not productively infect LSECs but RNA from HCV was recently shown to induce the expression of inflammatory cytokines and chemokines in human microvascular endothelial cells via TLR3 activation (56), indicating that LSECs can be activated through PRR engagement by HCV-derived products.

The "core" protein of HCV and HBV was reported to bind to TLR2 and induce TLR2-dependent inflammatory cytokine response in monocytes and macrophages (57, 58). Thus, one could presume that core proteins could also promote proinflammatory activation of LSECs via TLR2, aggravating hepatic inflammation. In this regard, a high correlation between TLR2 expression and hepatic inflammation and necrosis was demonstrated in the liver of HCV-infected patients (59).

**FUNDING INFORMATION**

This work was granted by NSERC of Government of Canada (grant 2895-2009 to Lucie Lamontagne) and Christian Bleau was supported by a NSERC fellowship. The funders have
no implication in study design, data collection and interpretation, or the decision to submit
the work for publication

ACKNOWLEDGMENTS

The authors want to acknowledge Corentine Lux, Pascale Bellaud and Eric Massicotte for
their technical assistance.
REFERENCES


virus or poly(I:C) induce IL-33 in hepatocytes in murine models of hepatitis. PLoS One 8:e74278.


Figure 1: Hepatic damages and viral replication in highly hepatotropic MHV3- and attenuated 51.6- and YAC-MHV3-infected mice. Groups of 5 or 6 C57BL/6 were intraperitoneally infected with 1000 TCID₅₀ (tissue culture infective dose 50%) of MHV3, 51.6-MHV3 or YAC-MHV3. (A) Histopathological analysis was conducted on livers from mock- and viral-infected mice from each group at 24 and 72 h p.i. Inflammatory and necrosis foci are indicated by arrows. (B) Summary of occurrence of necrotic and inflammatory foci in livers from infected mice at 24, 48 and 72 h p.i. ALT (C) and AST (D) activities were assayed in serum samples from mock- and viral-infected mice at 24, 48 and 72 h p.i. MHV3 replication in livers from each group of infected mice was determined by analysis of the nucleoprotein (NP) RNA expression at 24, 48 and 72 h p.i. by RT-qPCR (E) and by viral titration (TCID₅₀) (D) at 24 h and 72 h p.i. Values represent fold change in gene expression relative to mock-infected mice after normalisation with HPRT expression. Arrows indicate inflammatory or necrosis foci. Values are means plus standard errors of the mean (error bars). ***P < 0.001 when compared with mock-infected mice and ††P < 0.01; †††P < 0.001 when compared with MHV3-infected group.

Figure 2: Gene expression and/or production of Caveolin-1, Fgl2 and IL-33 in the liver of MHV3-, 51.6-MHV3- and YAC-MHV3-infected mice. Groups of 5 or 6 C57BL/6 (WT) were intraperitoneally infected with 1000 TCID₅₀ (tissue culture infective dose 50%)
of MHV3, 51.6-MHV3 and YAC-MHV3. At 24, 48 or 72 h p.i., livers were collected from mock- and viral-infected mice of each group. mRNA expression for (A) caveolin-1, (C) Fgl-2, and (D) IL-33 genes was evaluated by qRT-PCR. Values represent fold change in gene expression relative to mock-infected mice (arbitrarily taken as 1) after normalisation with HPRT expression. In situ expression of caveolin-1 (B) and IL-33 (F) were determined by immunohistochemistry in livers from mock-, MHV3- and 51.6-MHV3-infected mice at 48 h p.i. Caveolin-1 and IL-33 positive cells are indicated by arrows. Production levels of IL-33 (E) were quantified by ELISA at 72 h p.i. in the liver of each mouse. Immunolocalization of IL-33 in LSECs (G) was confirmed by double immunostaining of IL-33 (green) and CAV-1 (red) in livers from mock- and MHV3-infected mice at 48 and 72 h p.i. Cell nuclei were counterstained with Hoescht (blue). In situ expressions of caveolin-1 (B) or IL-10 and caveolin-1 (G) by endothelial cell are indicated by arrows. Values are means plus standard errors of the mean (error bars). *P < 0·05; **P < 0·01; ***P < 0·001 when compared with mock-infected mice, and †P < 0·05; ††P < 0·01; †††P < 0·001 when compared with MHV3-infected group.

Figure 3: Gene expression and production of IL-10, TGF-β, IL-6, TNF-α, CXCL1, CCL2 and CXCL10 in the liver of MHV3-, 51.6-MHV3- and YAC-MHV3-infected mice. Groups of 5 or 6 C57BL/6 mice were intraperitoneally infected with 1000 TCID₅₀ (tissue culture infective dose 50%) of MHV3, 51.6-MHV3 and YAC-MHV3. At 24, 48 or 72 h p.i., livers were collected from mock- and viral-infected mice of each group. Section I: (A) IL-10, (C) TGF-β, (E) IL-6, and (G) TNF-α mRNA fold changes were analyzed by
qRT-PCR. Values represent fold change in gene expression relative to mock-infected mice (arbitrarily taken as 1) after normalisation with HPRT expression. Production levels of (B) IL-10, (D) TGF-β, (F) IL-6, and (H) TNF-α were quantified by ELISA test at 72 h p.i. in the liver of each mouse. Section II: *In situ* expression of IL-10 and caveolin-1 was assayed by immunohistochemistry in livers of mock-, MHV3- and 51.6-MHV3-infected mice at 48 h p.i. (arrows show IL-10 and Caveolin-1-expressing endothelial cells)  
Section III: (A) CXCL1, (C) CCL2, and (E) CXCL10 mRNA fold changes were analyzed by qRT-PCR. Values represent fold change in gene expression relative to mock-infected mice (arbitrarily taken as 1) after normalisation with HPRT expression. Production levels of (B) CXCL1, (D) CCL2, and (F) CXCL10 were quantified by ELISA at 72 h p.i. in the liver of each mouse. Values are means plus standard errors of the mean (error bars). *P* < 0·05; **P** < 0·01; ***P*** < 0·001 when compared with mock-infected mice, and †*P* < 0·05; ††*P* < 0·01; †††*P* < 0·001 when compared with MHV3-infected group.  

**Figure 4: Gene expression of TLR2, 3, 4, 7 and helicases RIG-I and MDA5 in the liver of MHV3-, 51.6-MHV3- and YAC-MHV3-infected mice.** Groups of 5 or 6 C57BL/6 (WT) were intraperitoneally infected with 1000 TCID₅₀ (tissue culture infective dose 50%) of MHV3, 51.6-MHV3 and YAC-MHV3. At 24, 48 or 72 h p.i., livers were collected from mock- and viral-infected mice of each group. mRNA expression for (A) TLR2, (B) TLR3, (C) TLR4, (D) TLR7, (E) RIG-I and (F) MDA5 genes was evaluated by qRT-PCR. Values represent fold change in gene expression relative to mock-infected mice (arbitrarily taken as 1) after normalisation with HPRT expression. Values are means plus standard errors of the
mean (error bars). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared with mock-infected mice, and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$ when compared with MHV3-infected group.

**Figure 5: Percentages and numbers of intrahepatic mononuclear cell subsets in livers from MHV3-, 51.6-MHV3 and YAC-MHV3-infected mice.** Intrahepatic mononuclear cells were isolated from groups of 5 or 6 mock-infected or MHV3-, 51.6-MHV3 and YAC-MHV3-infected C57BL/6 mice at 24 and 48 h p.i., immunolabeled with NK1.1, CD3, Gr1, CD11b, CD19, CD4 and CD8 monoclonal antibodies and analyzed by cytofluorometry. Percentages of (A) NKT (NK1.1+CD3+), (B) NK (NK1.1+CD3), (C) neutrophils (Gr1hi CD11bhi), (D) macrophages (Gr1+ CD11bint) cells, (E) CD19 (CD3+CD19+), (F) CD4 (CD3+CD4+) and (G) CD8 (CD3+CD8+) were evaluated in livers from each group of infected mice (section I). Absolute numbers for each cell subset (calculated in using respective percentages reported to total number of isolated mononuclear cells) were similarly recorded in livers of respective groups (section II). Values are means plus standard errors of the mean (error bars). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared with mock-infected mice, and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$ when compared with MHV3-infected group.

**Figure 6: Permissivity of LSECs to MHV3, 51.6-MHV3 and YAC-MHV3 infection.** Mouse LSECs were isolated by Percoll gradient and enriched by immunomagnetism with anti-CD146 antibodies. (A) LSECs were characterized by immunolabelling with antibodies for CD146, CD54, CD31 and F4/80 cell markers and cyrofluorometric analysis. B-C)
LSECs were infected at 0.1 MOI of MHV3, 51.6-MHV3 and YAC-MHV3 and the evolution of cytopathic effects was noted in LSEC cultures up to 5 days p.i. (B). The kinetics of MHV infections were monitored by quantifying viral titers in supernatants of infected LSECs (C). All experiments were conducted in triplicate. Results are representative of two independent experiments. Values are means for each point. †††P < 0·001 when compared with MHV3-infected cells.

Figure 7: Expression levels of Fgl2, IL-33 and caveolin-1, and production of NO in *in vitro* MHV3-, 51.6-MHV3 and YAC-MHV3-infected LSECs. LSECs were infected at 0.1 MOI of MHV3, 51.6-MHV3 and YAC-MHV3 and RNA and supernatant from LSECs infected with each viral strain were collected at 24, 48 or 72 h p.i. (A) IL-33, (C) Fgl-2, and (E) caveolin-1 mRNA fold changes were analyzed by qRT-PCR. Values represent fold change in gene expression relative to uninfected LSECs (control arbitrarily taken as 1) after normalisation with HPRT expression. All samples were run in duplicate. (B) Production levels of IL-33 were quantified by ELISA and NO levels (D) were assayed by the Griess reaction in supernatants at 48 h p.i. All experiments were run in duplicate and results are representative of two independent experiments. Values are means plus standard errors of the mean (error bars). *P < 0·05; **P < 0·01; ***P < 0·001 when compared with mock-infected cells, and †P < 0·05; ††P < 0·01; †††P < 0·001 when compared with MHV3-infected cells.
Figure 8: Gene expression and production of IL-10, TGF-β, IL-6, TNF-α, CXCL1, CCL2 and CXCL10 in in vitro MHV3-, 51.6-MHV3- and YAC-MHV3-infected LSECs. LSECs were infected at 0.1 MOI of MHV3, 51.6-MHV3 and YAC-MHV3 and RNA and supernatant from LSECs infected with each viral strain were collected at 24, 48 or 72 h p.i. Section I: (A) IL-10, (C) TGF-β, (E) IL-6, and (G) TNF-α mRNA fold changes were analyzed by qRT-PCR. Values represent fold change in gene expression relative to uninfected LSECs (control arbitrarily taken as 1) after normalisation with HPRT expression. All samples were run in duplicate. Production levels of (B) IL-10, (D) TGF-β, (F) IL-6, and (H) TNF-α were quantified by ELISA in supernatants at 24 h p.i. Section II: (A) CXCL1, (C) CCL2, and (E) CXCL10 mRNA fold changes were analyzed by qRT-PCR. Values represent fold change in gene expression relative to control (uninfected) LSECs after normalisation with HPRT expression. All samples were run in duplicate. Production levels of (B) CXCL1, (D) CCL2, and (F) CXCL10 were quantified by ELISA in supernatants at 24 h p.i. All experiments were conducted in duplicate and results are representative of two independent experiments. Values are means plus standard errors of the mean (error bars). *P < 0·05; **P < 0·01; ***P < 0·001 when compared with mock-infected cells, and †P < 0·05; ††P < 0·01; †††P < 0·001 when compared with MHV3-infected cells.

Figure 9: Role of TLR2 in viral replication and expression of IL-6 and CXCL1 in MHV3- 51.6-MHV3- and YAC-MHV3-infected LSECs. A) LSECs were infected at 0.1 MOI of MHV3, 51.6-MHV3 and YAC-MHV3. At 24, 48 and 72 h p.i., RNA from infected LSECs was extracted and mRNA expression levels for TLR2 gene was determined by qRT-
PCR. B to D) LSECs were treated with specific siRNA against TLR2 prior to infection with viruses or treatment with the specific TLR2 agonist Pam3Cys (as positive control) for 24h. (B) MHV nucleoprotein (MHV-N), (C) IL-6, and (D) CXCL1 mRNA expression levels were determined by qRT-PCR. Values represent fold change in gene expression relative to control (uninfected) LSECs after normalisation with HPRT expression. All samples were run in duplicate and results are representative of two independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared with ctrl cells, and †$P < 0.05$ when compared with MHV3-infected cells.

**Figure 10: Mortality, hepatic damages and viral replication in MHV3- and 51.6-MHV3-infected C57BL/6 (WT) and TLR2 KO mice.** Groups of 6 or 7 C57BL/6 (WT) and TLR2 KO mice were intraperitoneally (i.p.) infected with 1000 TCID$_{50}$ (tissue culture infective dose $50\%$) of MHV3 and 51.6-MHV3. Percentages of (A) MHV3- and (B) 51.6-MHV3-infected surviving mice were recorded at various times post-infection (p.i.). (C) Histopathological analysis was conducted on livers from mock-, MHV3- and 51.6-MHV3-infected WT and TLR2 KO at 72 h p.i. Necrosis foci are indicated by arrows (D) MHV3 and 51.6-MHV3 replication in livers from infected WT and TLR2 KO mice was determined by viral titration (TCID$_{50}$) at 24 h and 72 h p.i. Values are means plus standard errors of the mean (error bars). Results are representative of two different experiments. ***$P < 0.001$ when compared with WT mice.
Figure 11: Expression of IL-10 and Caveolin-1 in livers from MHV3-infected TLR2 KO mice. Groups of 6 TLR2 KO mice were intraperitoneally (i.p.) infected with 1000 TCID_{50} (tissue culture infective dose 50%) of MHV3 and immunolocalization of IL-10 in the liver and ECs of mock- and MHV3-infected TLR2 KO mice was determined by double immunostaining of IL-10 (green) and Caveolin-1 (CAV) (red) at 48 h p.i. Cell nuclei were counterstained with Hoescht (blue). *In situ* expressions of IL-10 and caveolin-1 by endothelial cell are indicated by arrows.
Figure 1

Mock 24h p.i. Virus Time p.i. (hrs) Inflammatory foci Hepatic necrosis foci
MHV3 24 ++ - 72 + ++++
48 ++ ++
YAC-MHV3 24 + - 72 + -
48 + -
51.6-MHV3 24 - - 72 + -
48 + +

+ (scarse foci); ++ (10% of hepatic tissue); +++ (20-30% of hepatic tissue);
++++ (up to 40% of hepatic tissue)

**Figure 1**
Figure 2
Figure 3
(section I)
Figure 3
(Section II)
Figure 3

(Section III)

A. CXCL1

B. CXCL1

C. CCL2

D. CCL2

E. CXCL10

F. CXCL10

Figure 3

(Section III)
Figure 4
Figure 5
(Section I)
Figure 5
(Section II)
Figure 6

A

CD146

CD54

CD31

F4/80

90.1%

91.3%

86.6%

3.0%

Events

B

Cytopathic effects

MHV3

51.6-MHV3

YAC-MHV3

% cell lysis

24 48 72 96 120

Time postinfection (hrs)

TCID50 (Log10)

24 48 72 96 120

Time postinfection (hrs)

Viral titers

†††

†††

†††

†††
Figure 7
Figure 8
(section I)
Figure 8
(section II)
Figure 9

A. TLR2

B. MHV-N

C. IL-6

D. CXCL1

E. IL-10

mRNA fold change over time post-infection with different conditions.
Survival curve of MHV3-infected mice

Survival curve of 51.6-MHV3-infected mice

Viral titers in the liver

Figure 10
Figure 11
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>5'-GAAAGACTTGCTCGAGATGTGATG-3'</td>
<td>5'-CACACAGAGGGCCACAATGT-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-TCGGAGGCTTAAATTACATGTGGTC-3'</td>
<td>5'-TGCCATTGCAAACTCTTTTCT-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-TCCCAGGTCTCTTCAAGGGGA-3'</td>
<td>5'-GGTGAGGAGCAGATGTCCGG-3'</td>
</tr>
<tr>
<td>CCL2</td>
<td>5'-GCAGCAGGTGGCCCAAAGGA-3'</td>
<td>5'-GGTCAGGAGACCATCTCTCTTG-3'</td>
</tr>
<tr>
<td>CXCL10</td>
<td>5'-GGCCATAGGGGAAGCTGAAAT-3'</td>
<td>5'-TGCGGGAATCTCTCAACAC-3'</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5'-CTCCGCTGCTGTTTGGAGAACT-3'</td>
<td>5'-CGGAACGAATACACCGGTGAT-3'</td>
</tr>
<tr>
<td>TLR3</td>
<td>5'-TGCGGCTGAAATGGGAGAACATCT-3'</td>
<td>5'-TGCGGAAATCAGGAGGT-3'</td>
</tr>
<tr>
<td>CAV-1</td>
<td>5'-GCACACCAAGGGAGATTG-3'</td>
<td>5'-CACGTCGCTGTTGAGATGCT-3'</td>
</tr>
<tr>
<td>TLR7</td>
<td>5'-CAGTGAACCTCTGGCCGTTGA-3'</td>
<td>5'-CAAGCCCCGTTGCTGAGAA-3'</td>
</tr>
<tr>
<td>MHV-N</td>
<td>5'-TGAAGGTCTGACCGGCTGCA-3'</td>
<td>5'-TTGGCCCCACGGGATG-3'</td>
</tr>
<tr>
<td>RIG-I</td>
<td>5'-GCCCCAGTGAAGCTTCAAGTCAGTCAG-3'</td>
<td>5'-GAGAAACAGATGCTGCTGTC-3'</td>
</tr>
<tr>
<td>MDA-5</td>
<td>5'-GCCCTCTCTCCTCTCGAGACT-3'</td>
<td>5'-GCTGGAGGAGGTCAGCAA-3'</td>
</tr>
<tr>
<td>IL-33</td>
<td>5'-GCTGCGTCTGGACACATTG-3'</td>
<td>5'-GGGAGGCAAGACTGTTGAA-3'</td>
</tr>
<tr>
<td>Fgl2</td>
<td>5'-CTGTGTGCTCAACAGGTTGGA-3'</td>
<td>5'-GATGTGAACCGCTGTAAGCT-3'</td>
</tr>
<tr>
<td>CXCL1</td>
<td>5'-CGCAAGATGCATAGCCACACTCA-3'</td>
<td>5'-CAAGGGAGCTGCTGAGGTC-3'</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-GATGCCCCAGGGAGAA-3'</td>
<td>5'-CAACCCAGGAATTCAAATG-3'</td>
</tr>
<tr>
<td>TGF-β</td>
<td>5'-AGCGCTACTGTCTTGTGA-3'</td>
<td>5'-GCTGATCCCCGTGATTTCCA-3'</td>
</tr>
</tbody>
</table>
Table II. Transcription levels of several genes in liver from MHV3- and 51.6-MHV3-infected C57BL/6 (WT) and TLR2 KO mice at 72 h p.i.

<table>
<thead>
<tr>
<th>Genes</th>
<th>MHV3</th>
<th>51.6-MHV3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>TLR2 KO</td>
</tr>
<tr>
<td>TNF-α</td>
<td>70.2 ± 7.2</td>
<td>44.9 ± 4.0 ***</td>
</tr>
<tr>
<td>IL-6</td>
<td>43.5 ± 5.7</td>
<td>11.2 ± 5.0 ***</td>
</tr>
<tr>
<td>IL-10</td>
<td>5.8 ± 1.4</td>
<td>44 ± 11.7 ***</td>
</tr>
<tr>
<td>CXCL1</td>
<td>131 ± 15</td>
<td>21.1 ± 8.7 ***</td>
</tr>
<tr>
<td>CCL2</td>
<td>1027 ± 134</td>
<td>155 ± 37 ***</td>
</tr>
<tr>
<td>CXCL10</td>
<td>213 ± 20</td>
<td>69 ± 15 ***</td>
</tr>
<tr>
<td>Fgl2</td>
<td>6.65 ± 0.70</td>
<td>10.1 ± 1.2</td>
</tr>
<tr>
<td>IL-33</td>
<td>4.5 ± 0.4</td>
<td>3.7 ± 0.3</td>
</tr>
</tbody>
</table>

Groups of 5 or 6 C57BL/6 (WT) or TLR2 KO mice were intraperitoneally infected with 1000 TCID50 (tissue culture infective dose 50%) of MHV3 or 51.6-MHV3. At 72 h p.i., livers were collected from mock- and viral-infected mice from each group and mRNA fold changes for several genes were analyzed by qRT-PCR. Values represent fold change in gene expression relative to mock-infected mice after normalisation with HPRT expression. Samples from each mouse were run in duplicate. Values that are significantly different between MHV3-infected TLR2 KO and C57BL/6 (WT) mice or between 51.6-MHV3-infected TLR2 KO and C57BL/6 (WT) are indicated by asterisks as follows: ***$P < 0.001$  *$P < 0.05$. n.d. not detectable.