Innate immune responses in viral hepatitis:

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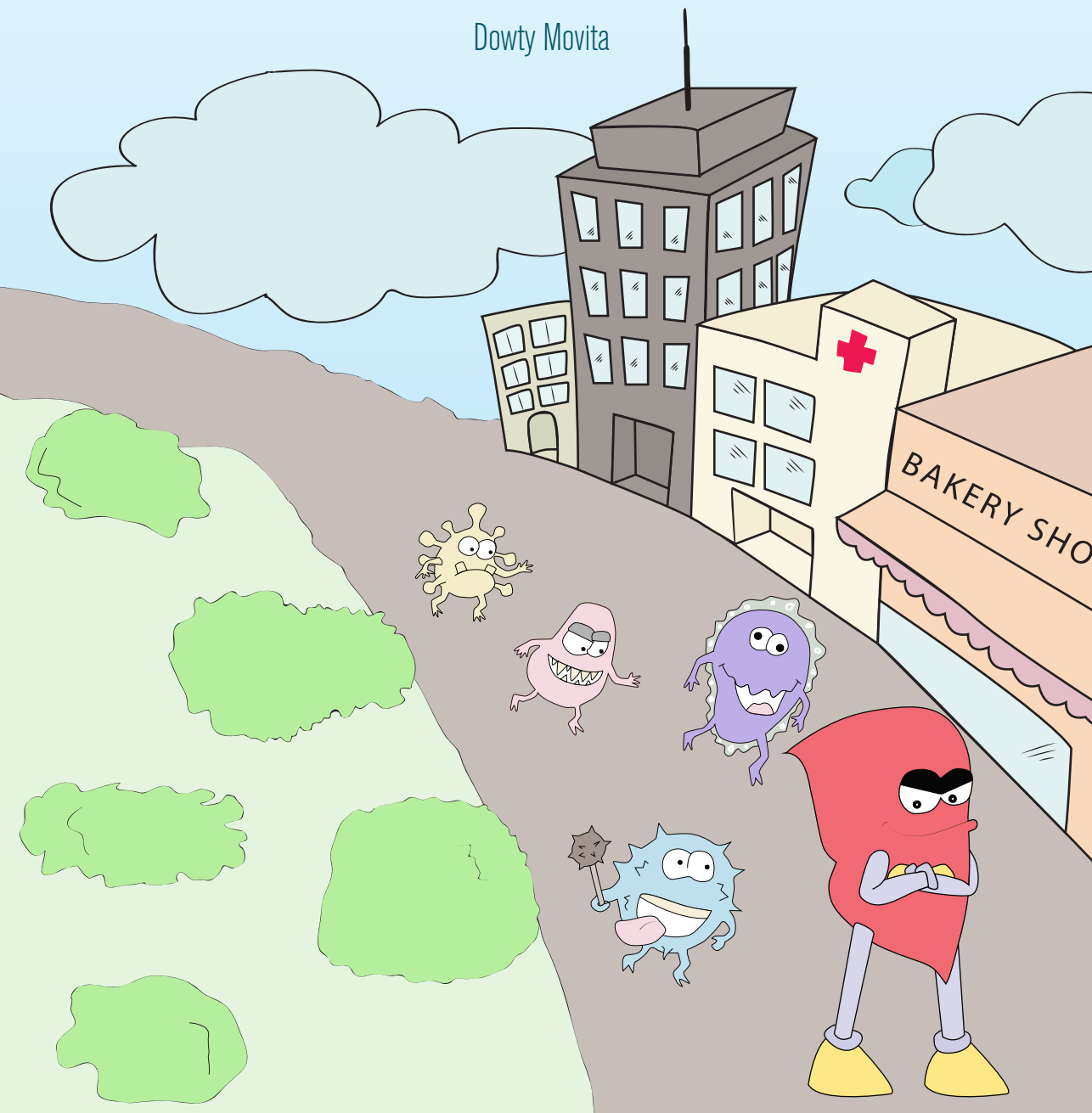

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Innate Immune Responses in Viral Hepatitis: the role of Kupffer cells and liver-derived monocytes in shaping intrahepatic immunity in mice using the LCMV infection model

Niet-specifieke afweerreacties in virale hepatitis:
De rol van Kupffer cellen en lever-monocyten in de vorming van intrahepatische immuniteit in muizen gebruikmakend van het LCMV infectiemodel

Thesis

to obtain the degree of Doctor from the
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List of abbreviations

CLR	C-type lectins
CTCF	CCCTC-binding factor
DAB	diaminobenzidine
DC	dendritic cells
DHR	dihydrorhodamine
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
HBV	Hepatitis B virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B early antigen
HBsAg	Hepatitis B surface antigen
HCC	hepatocellular carcinoma
HCV	Hepatitis C virus
HO-1	heme-oxygenase 1
HRP	horse radish peroxidase
HSPG	heparan sulfate proteoglycan
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p	intraperitoneal
IRAK-M	interleukin-1 receptor-associated kinase M
IRF3	interferon regulatory factor 3
ISG	interferon-stimulated gene
i.v	intravenous
KC	Kupffer cells
LCMV	Lymphocytic choriomeningitis virus
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
LysM	lysozyme M
MARCO	macrophage receptor with collagenous structure
MCMV	Murine cytomegalovirus
MHC II	major histocompatibility complex class II
MHV	Mouse hepatitis virus
MR	mannose receptor
NK cells	natural killer cells
NLR	NOD-like receptor
NPC	non-parenchymal cells
OAS	oligoadenylate synthetase
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
RLR	RIG-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
SEM	standard error of the mean
SR	scavenger receptor
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TGFβ	transforming growth factor beta

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General introduction and outline of the thesis

Hepatitis B and C virus infection

Characteristics of hepatitis B and C infection

The hepatitis B virus (HBV) and hepatitis C virus (HCV) have infected more than 500 million people worldwide. Infections by HBV and HCV often persist and lead to liver complications such as cirrhosis or hepatocellular carcinoma (1-5). While HBV can be cleared in 95% of infected adults, chronic infection is seen in 90% of neonates exposed to this virus (1, 2). In contrast, 70-90% of HCV-infected individuals develop chronic infection (4, 5). Currently, a highly effective vaccine against HBV, but not HCV, infection is available (6). Issues with HBV and HCV remain since heterogenous responses are observed in chronically infected patients to currently available therapies such as nucleoside analogues, interferon (IFN)-alpha, and ribavirin (2-4).

HBV and HCV belong to the hepadnavirus and flavivirus families, respectively (1-4, 7). HBV contains a 3.2 kb partially double-stranded DNA, of which the four open reading frames encode for five HBV proteins: polymerase (HBpAg), envelope or surface (HBsAg), core (HBcAg), early or pre-core (HBeAg), and X (HBxAg). Viral DNA is encapsulated by core protein, which is surrounded by an envelope, forming a complete viral or Dane particle (3, 8). In contrast to HBV, HCV contains a genomic 9.6 kb positive-strand RNA. Upon cellular entry, genomic HCV RNA is directly translated, resulting in a polyprotein of 3010 amino acids. This polyprotein is further processed and produces core protein, two envelop proteins (E1 and E2), and NS1, NS2, NS3, NS4A, NS4B, NS5A, and NS5B non-structural proteins that are important in viral replication (9-25).

HBV and HCV predominantly replicate in liver parenchymal cells, i.e. hepatocytes. The mechanisms of entry used by HBV or HCV are not completely elucidated, however several receptors for these viruses have been identified. Entry receptors for HBV include endonexin II (26), IL-6 (27), annexin V (28), apolipoprotein H (29), transferrin receptor (30), and gp180/carboxypeptidase D in the case of DHBV (31, 32) and sodium taurocholate cotransporting polypeptide (NTCP) (33). For HCV, claudin (34), CD81 (35, 36), human scavenger receptor class B type I (SR-B1) (37), dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) (38, 39), and liver and lymph node-specific intercellular adhesion molecule-3-grabbing non-integrin (L-SIGN) (38, 40) have been shown to be essential for cellular entry. Some of these receptors, e.g. SR-B1, DC-SIGN and L-SIGN, are also expressed in non-hepatocyte cells, and therefore they are regarded as entry co-receptors.

HBV and HCV infection display distinct mechanisms of replication. Following entry, HBV nucleocapsids are released into the cytoplasm, followed by release of the viral DNA genome into the nucleus and formation of the viral transcriptional template (termed covalently closed circular (ccc) DNA). Upon transcription, viral RNA transcripts are transported to the cytoplasm to be translated

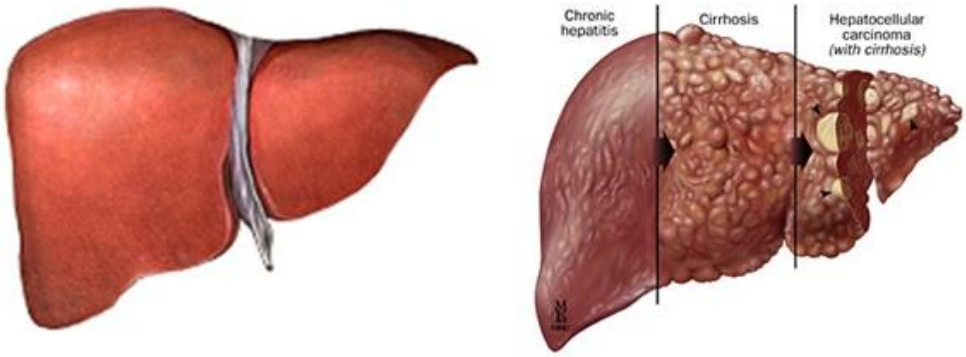

Figure 1. Liver pathology induced by HBV or HCV infection. *Left*, the morphology of healthy liver. *Right*, the progression of liver damage, from chronic hepatitis, liver cirrhosis and eventually hepatocellular carcinoma, induced by HBV or HCV infection.

into structural and nonstructural viral proteins. Upon encapsulation, this transcript also serves as pre-genomic RNA, which produces a circular double-stranded DNA genome by reverse transcription. Viral capsids, containing double-stranded DNA, further unite with the viral envelope proteins in the endoplasmic reticulum, bud off into the lumen, and exit the cell as virions (3, 41, 42). Distinct to the HBV replication, upon binding and receptor-mediated endocytosis, HCV virions are uncoated in the cytoplasm and the genomic RNA either undergo IRES-mediated translation followed by polyprotein processing, or replication. New virions containing genomic RNA and viral proteins, are generated in membranous compartment in the cytoplasm and released upon vesicle fusion with the plasma membrane (43). Differences in the HBV and HCV life cycle, as demonstrated by the fact that the double-stranded HBV DNA genome is completely concealed within capsid particles, whereas the double-stranded HCV RNA genome is freely exposed in the cytoplasm of the infected cell, resulting in distinct early innate defense mechanisms induced by these two viruses.

Intrahepatic innate immunity induced by HBV and HCV

The liver, as the largest organ in the body, is constantly exposed to a mix of arterial blood rich in oxygen and various gut-derived commensal microbes and food-borne antigens from the portal circulation. These conditions have led to the concept that the liver is immunologically tolerant under steady state conditions (44-46). However, in the HBV and HCV infection, hepatocytes are the main target of infection, supporting viral replication. This localized viral replication renders an accumulation of viral particles or antigens, able to activate the hepatocytes as well as immune cells, in the liver. This suggests that the liver is activated and may play an essential role in controlling the outcome of HBV and HCV infection.

Pathogenesis of HBV and HCV infections are determined by host and virus interactions. So far, progress in immunological studies on HBV and HCV in the liver has been impeded due to the limited host range of these viruses and the lack of small-animal models that are susceptible to HBV or HCV infection. Essential information regarding the mechanisms of intrahepatic immunity induced by HBV and HCV has been mainly provided by chimpanzee studies (1, 3, 5, 47).

The first line immune defense against HBV and HCV infection is represented by innate intrahepatic cells. Contributing to this are hepatocytes, liver sinusoidal endothelial cells (LSEC), Kupffer cells, plasmacytoid dendritic cells, natural killer (NK) cells. These intrahepatic cells recognize HBV and HCV, or their associated antigens, via their innate sensors as non-self and, either by induction of local antiviral defenses in the infected cell or by responding to the inflammatory microenvironment, activate intrahepatic immunity to recruit immune cells, both innate and adaptive, and modulate their actions (48-51). Particularly, LSEC, Kupffer cell and dendritic cells have the ability to either produce cytokines upon stimulation or may present viral antigen to activate T cells (52-54). Although clearance of HBV and HCV infection is executed by multi epitope-specific adaptive $CD4^+$ T, $CD8^+$ T and B cell responses (55-58), these responses are dependent and shaped by the early immunological events provided by innate immune cells in the liver (58, 59).

Intrahepatic cells are equipped with various pattern recognition receptors (PRRs) able to recognize specific pathogen-associated molecular pattern (PAMP) and sense viruses as foreign invaders. Toll-like receptor (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors, RIG-I and melanoma differentiation antigen 5 (MDA-5), and Nod-like receptors (NLRs) mediate virus sensing from the endosomal and cytosomal compartments to initiate innate defenses. In addition to these classical PRRs, several nucleic acid–binding proteins can also engage viral nucleic acid, and upon interaction with a particular PRR, act as receptors which activate signaling pathways. For example, Protein Kinase R (PKR) binds double-stranded RNA and further interacts with MAVS to activate the NF-kB signaling pathway (60, 61). Ligation of these PRRs results in the production of innate antiviral mediators, which can limit virus replication or provide chemotactic signals to recruit immune cells to the liver.

Due to the distinct characteristics of the HBV and HCV life cycles, activation of liver cells by these viruses is not identical. HBV infection in the liver is typically characterized by minimal intrahepatic immune activation (62). In contrast to the weak intrahepatic immune induction by HBV, upon entry, HCV is detected by intrahepatic viral sensors and induces transcription of various IFN-inducible genes (ISG) such as 2-5-oligoadenylate synthetase (2,5-OAS) and MxA, which are known to exhibit antiviral activity (49, 63, 64). However, this induction of antiviral activity is insufficient to completely eliminate the virus. Activation of IRF-1 and IRF-3 results in the induction of IFNα and

IFNβ mRNA (65-68). A viral inhibitory mechanism, provided by HCV NS3/4A, has been described to be responsible for limiting TLR3- and RIG-I-induced signaling pathways to produce IFNβ (69-73). Additionally, HCV E2 and NS5A have been shown to interfere with PKR binding to double-stranded RNA, resulting in the inhibition of IRF-1 phosphorylation (74-76); and NS3 has been shown to prevent the phosphorylation, dimerization and nuclear translocation of IRF-3 (77). Interestingly, although HCV NS3/4A, E2 and NS5A inhibit the expression of ISG, genomic analysis on HCV-infected livers of chimpanzees shows that there is a positive correlation between systemic viral load and the intrahepatic levels of various ISG (49, 63, 64), suggesting that evasion mechanisms are delivered by HCV to avoid the antiviral activity of type I IFN. It is postulated that these evasion mechanisms result in reduced sensitivity towards IFN's antiviral activity, and might explain why the increased intrahepatic levels of ISG cannot limit HCV replication.

Lymphocytic choriomeningitis virus (LCMV) infection model

A prototype model of persistent viral infection

The concept of persistent viral infection evolved from an observation in the LCMV Traub-infected mice which, due to *in utero* or perinatal infection, did not succumb to death due to the infection or cleared the infection. Ever since, LCMV infection in mice has been used as a preferred model to investigate immunological events during persistent viral infection characterized by high viral load levels. Although rodents are the natural host of LCMV, human infection by this virus has also been reported. Currently, 2 LCMV strains, Armstrong and clone 13, are predominantly used as prototypic viruses in studies on acute and persistent infection. LCMV clone 13 and Armstrong virus genomes differ by only 5 nucleotides, which translate into 2 amino acids (78, 79). The 2 amino acid difference in the viral "polymerase" and "glycoprotein" polypeptides is responsible for the distinct infection profiles of these 2 strains. Infection of C57Bl/6 mice with LCMV Armstrong is acute and results in viral clearance within 8 days after infection. The systemic viral load reaches its peak at 3-4 days after infection, followed by a complete clearance at day 8. In this case, viral clearance is mediated by a strong adaptive immune response characterized by proliferation and activation of highly effective LCMV-specific $CD4^+$ and $CD8^+$ T cells (80-82). In contrast to the acute, resolving infection induced by LCMV Armstrong, LCMV clone 13 induces persistent infection and high viral titers are observed weeks after inoculation of mice (83, 84). This persistent infection occurs due to lack of the maintenance of strong antiviral responses as demonstrated by ablation of specific T cell responses to multiple viruses, as well as antibody responses to many different antigens (84, 85). Immunosuppression in mice infected by LCMV clone 13 appears to result from a defect in antigen presentation, rather than from a direct effect on T cells and B cells (84-86). This concept, that a single amino acid

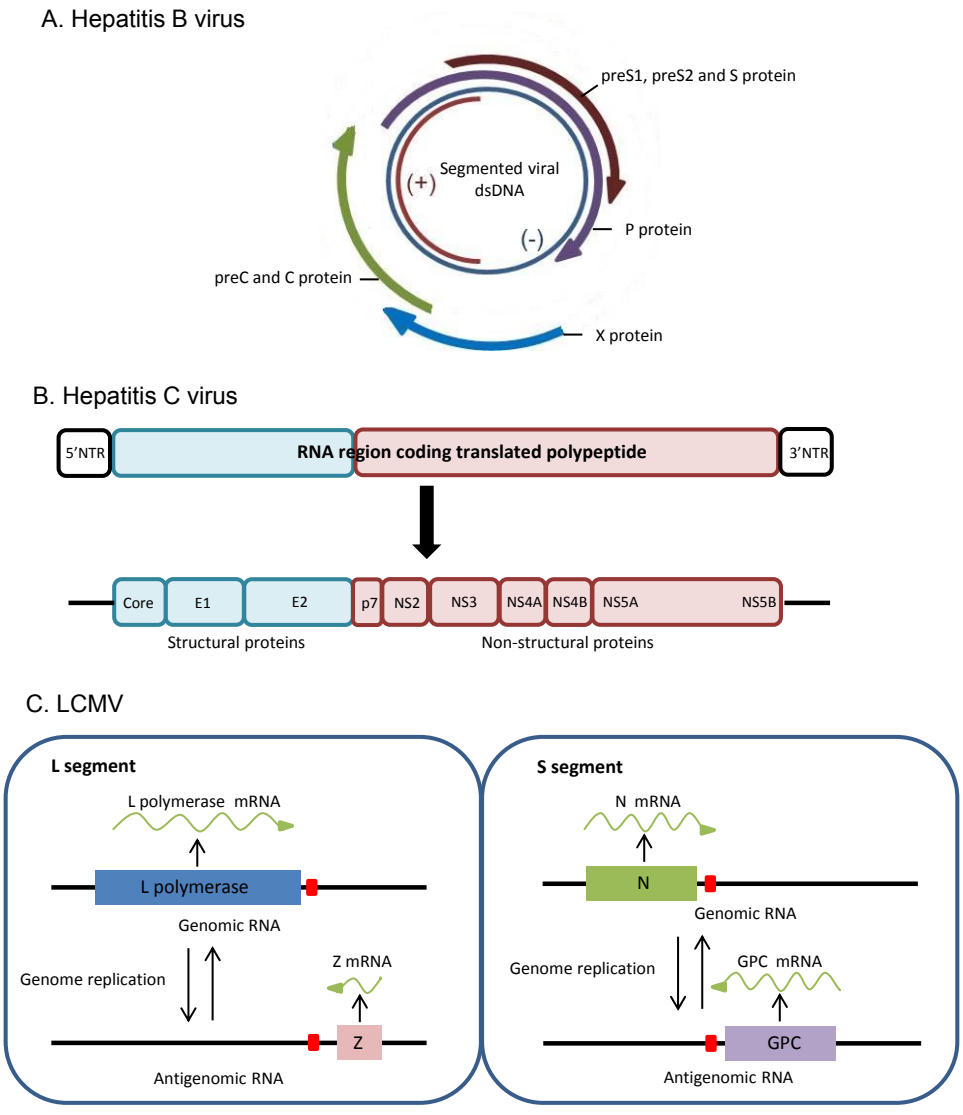

Figure 2. Genome organization of HBV, HCV and LCMV. A) HBV genome is a segmented double stranded DNA molecule, consisting of 4 major open reading frames (ORFs) encoding preC and C protein; preS1, preS2 and S protein; P protein and X protein. B) HCV genome is a positive single stranded RNA molecule encoding a polyprotein precursor of 3010 amino acids. HCV polyprotein is cleaved into 10 different products: core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. C) LCMV genome consists of 2 segmented negative single stranded RNA molecule: the L and S segment which are ambisense. L segment encodes for L polymerase and Z protein; whereas S segment encodes for GPC and N protein.

change in a pathogen can alter its infection characteristic (i.e. acute or chronic), is also observed by studies in HIV (87) and hepatitis E virus (88) infection.

Numerous key findings in adaptive immunity during persistent viral infection have been demonstrated using the LCMV infection model. These findings were later confirmed in human studies. First, MHC-restricted action of the cytotoxic T cell to kill the infected cells was demonstrated in LCMV infected mice (89-91). In these studies, recognition of both viral-derived peptide and MHC protein is shown to govern the specificity of cytotoxic T cell action. Furthermore, the importance of MHC-specific cytotoxic T cell action to rapidly clear an otherwise LCMV persistent infection was shown in an adoptive transfer experiment (92, 93). Second, LCMV studies contributed to the understanding of T cell mediated cell lysis, particularly perforin based cytotoxicity (94). By using perforin deficient mice, Kagi *et al.* showed that T cell mediated cytotoxicity to LCMV-WE infected cells were largely absent in these mice (95). Third, LCMV studies contributed to the emergence of the concept of memory in adaptive immunity. Using this model, it was demonstrated that T cells, in the second exposure and after the first LCMV and its antigen has been completely eliminated, have the ability to memorize their related antigens. The second antigen encounter rapidly and potently activates T cells responses (96). These findings were confirmed in human studies of yellow fever and small pox infection (97). In addition to the adaptive immune activation, several phenomena of adaptive immune suppression were also discovered in persistent LCMV-infected mice. The immune suppression is indicated by the absence of robust cytotoxic T cell response, characterized by decreased cytotoxic T cell activity, and reduced numbers of thymocytes (98-100). In addition, the effector T cells display an “exhausted” phenotype. Despite being activated, these cells upregulate inhibitory signals which diminish their effector functions (101, 102). This phenomenon is also observed in HIV, hepatitis B and hepatitis C infection (103-106).

In addition, a prominent finding in innate immunity, i.e. the feature of NK cells to selectively kill tumor cells (107) was also demonstrated using the LCMV infection model. Welsh *et al.* found that a population of cytotoxic cells appeared early after LCMV infection, even before the cytotoxic T cell response started (108). Furthermore, recently, the importance of NK cells as innate effectors to bridge innate and adaptive immune response has been demonstrated by its role in regulating $CD4^+$ T cells, which subsequently control $CD8^+$ T cells during viral infections (109).

Molecular and cell biology of arenavirus

LCMV are enveloped viruses with a bi-segmented negative-stranded RNA genome, belonging to the arenavirus family (110, 111). The RNA genome of approximately 11 kb is divided into S and L segment. Each genome segment is translated from both directions since the coding sequen-

ces are positioned in opposite orientations, divided by a non-coding intergenic region (IGR), which serves as the transcription termination signal. The S segment encodes for the GPC (glycoprotein precursor) and NP (nucleoprotein), and the L segment encodes for L polymerase (a viral RNA-dependent RNA polymerase (RdRp)) and a small Z protein. The NP and L coding regions are translated into a genomic-complementary mRNA from which proteins are generated. In contrast, an anti-genomic RNA species, acting as a replicative intermediates is used as a template to generate the GPC and Z mRNAs (112, 113). GPC is post-translationally cleaved by the cellular site 1 protease (S1P) into 2 mature virion glycoproteins GP1 and GP2. Trimers, consisting of GP1 and GP2, form spikes on the viral envelope. The life cycle of this virus is restricted to the cell cytoplasm.

The cellular receptor for LCMV is a-dystroglycan (a-DG) (114, 115). This receptor mediates interaction with viral GP1 and entry upon initial cellular attachment. LCMV virions are taken up in smooth-walled vesicles (116) and fusion between the viral and cell membranes is triggered by the acidic environment of the late endosome. The low pH of the endosome is predicted to trigger conformational changes in the arenavirus GP (117-119), exposing a fusogenic peptide that mediates fusion of the virion and host cell membranes (120, 121). Upon release of viral genomic RNA, protein synthesis and genomic RNA replication, formation and budding of arenavirus infectious progeny requires assembly of the viral ribonucleoproteins (RNPs) and the cellular membranes enriched with viral GPs, mediated by a matrix (M) protein. The Z protein, via its Z proline-rich late domain motifs, has been shown to be essential in determining virus budding (122-125).

Splenic macrophages, intrahepatic Kupffer cells and monocytes in the LCMV model

Interest in investigating innate immunity in the context of persistent infection is emerging, and the LCMV infection model serves as a suitable model for this purpose. In particular, LCMV clone 13 has been shown to increase its replication in macrophages (79). Splenic dendritic cells and liver Kupffer cells are among the first innate cells to become infected by LCMV, as evidenced by the presence of LCMV-NP protein in $F4/80^+$ and $CD11c^+$ cells 3 days after infection (84, 126). Following this early event, at day 5 post infection, hepatocytes are infected by LCMV (126). LCMV infection in the liver induces recruitment of monocytes from the circulation, as demonstrated by the intrahepatic increase of the $F4/80^+$ cells (127). Recent evidence indicates that circulating blood monocytes also support LCMV replication upon *intracerebral* infection at birth or congenital *in utero* infection. It is postulated that circulating immune cells supporting virus replication may serve as a virus reservoir to spread the infection, resulting in persistent infection (128).

In LCMV model, cytotoxic T cells have been recognized to play an important role in resolving viral infection. Huang et al recently showed the crucial role of monocytes in activating the adap-

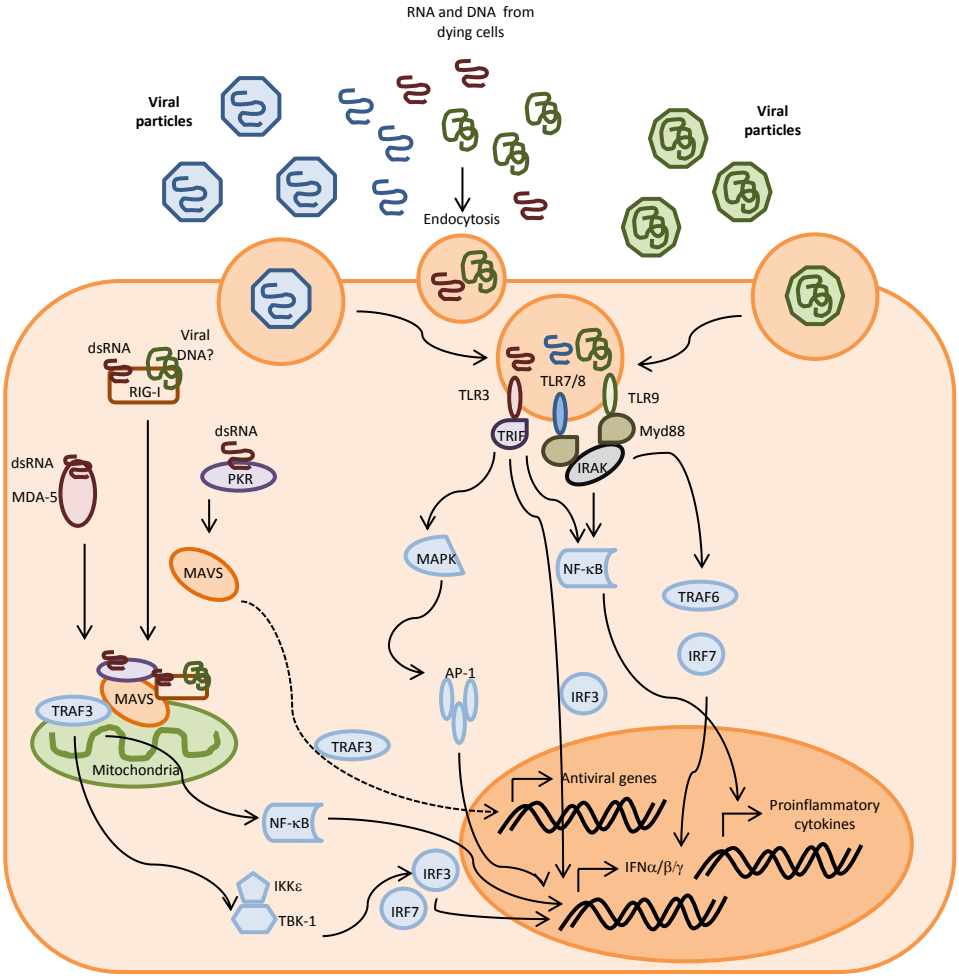

Figure 3. Innate activation by DNA or RNA viruses. Innate sensing of viruses occurs through the combined action of TLRs (TLR3, TLR7/8, TLR9), RIG-I, MDA-5 and PKR. These proteins recognize unique features of viral genome which leads to downstream signaling as indicated by the arrows. This results in the induction of antiviral and immunomodulatory genes.

tive immunity, resulting in the resolution of acute viral hepatitis. In the early phase of infection, LCMV-induced inflammatory monocytes form iMATEs (intrahepatic myeloid-cell aggregates for T cell population expansion) in the liver that support the proliferation of cytotoxic T cells (129). Formation of iMATEs is mediated by TNF and not accompanied with immunopathology. Although observed in the early phase of infection, iMATEs are absent in the chronic phase. Absence of iMATEs might contribute to the reduced proliferation and thus activity of cytotoxic T cell as observed in the persistent viral infection. This finding improves our knowledge in the establishment of exhaustion of the cytotoxic T cells. Additionally, splenic monocytes/ macrophages can also act as an immune

suppressor via IL-10 production. IL-10 has been identified as an important factor for the initiation and maintainance of chronic viral infection in the LCMV clone 13 infection (130). During LCMV infection, IL-10 is produced by dendritic cells and NK cells, amongst others. However, IL-10 produced by these cells does not contribute to the LCMV chronicity. In contrast, monocytes/ macrophages- and $CD4^+$ T cell-derived IL-10 has been shown to determine whether resolution or viral clearance takes place, as demonstrated by improved clearance of virus when monocyte/ macrophage- and $CD4^+$ T cell-derived IL-10 are depleted (131). Additionally, immune regulatory functions of Kupffer cells and monocytes in LCMV infection have been described. Inflammatory monocytes, which upon recruitment to the site of infection display a dendritic cell-like phenotype, perform hemophagocytosis (132). Similarly, Kupffer cells in the liver have been shown to perform viral phagocytosis (133). Both activities resulted in the reduction of cytotoxic T cell activity, and its subsequent tissue damage. These studies indicate that depending on the phase of infection, LCMV-induced inflammatory monocytes can perform distinct functions and contribute to either resolution or persistence of infection.

Toll-like receptors in viral hepatitis

TLR signaling pathways

Innate responses were previously considered to be nonspecific. This view changed following the discovery of a family of proteins similar to the *Drosophilla* Toll protein: TLRs; these serve as specific pattern recognition receptors. TLRs are a family of receptors that contain a leucine-rich repeat domain in the ectodomain mediating recognition of pathogen-associated molecular patterns (PAMPs), a transmembrane domain, and a Toll-IL-1 receptor (TIR)-containing intracellular domain mediating signal transduction. PAMPs recognition by TLRs results in activation of various transcription factors: NF-κB, AP-1 and IRFs. Recent advances in the field of TLR signaling have revealed the complex interaction between diverse adaptor proteins which differ for distinct TLR (134, 135). So far, 10 and 12 TLRs have been described for human and mice, respectively (136).

TLRs recognize a diverse set of ligands with some level of specificity. Ligation of the TLR receptor leads to the activation of the MyD88 and/or the TRIF signaling pathway. TLR1, TLR2, TLR5, TLR6, TLR7 and TLR9 signal through a MyD88-dependent pathway while TLR3 signals through a TRIF-dependent pathway. Interestingly, TLR4 uses both pathways. As demonstrated in 293 cells, unlike MyD88 or TIRAP, TRIF is involved in IFNβ promoter activation (137, 138). Both MyD88 and TRIF pathways require the presence of other accessory molecules. Mal (MyD88-adaptor like)/ TIRAP is crucial in the MyD88 pathway. Mal facilitates MyD88 delivery to the intracellular domain of activated TLR2 and TLR4. It also contains a PIP2-binding domain which recruits other Mal proteins to the plasma membrane. Mal is involved in downstream signaling of TLR1, TLR2, TLR4 and TLR6

pathway, but not of TLR3, TLR5, TLR7 and TLR9 (139-144). TRAM (TRIF-related adaptor molecule) is crucial in the TRIF pathway and is activated exclusively downstream of TLR4 ligation (145). TRAM is located at the plasma membrane and the Golgi apparatus, and co-localizes with TLR4. TRAM functions to recruit TRIF to the plasma membrane. While TRIF functions downstream of both TLR3 and TLR4, TRAM is only involved in TLR4 signaling via association with MyD88, Mal and TLR4 (146, 147). In addition to TRAM, Oshiumi et al. described TICAM-1 as an additional adaptor molecule in the TRIF pathway. It is downstream of TLR3 and activates IRF3 after poly I:C ligation. The function of TICAM-1 is closely associated with TICAM-2 that conveys signals from TLR3 to TICAM-1 (148, 149).

TLR-induced IFN production by innate immune cells

During viral infection, TLRs are predominantly triggered by viral single-stranded or double-stranded RNA or specific DNA motifs, leading to their activation (reviewed in Ref. (150)). TLR3, TLR7, TLR8 and TLR9 are the major PRRs that recognize distinct types of virally-derived nucleic acids and activate signaling cascades that result in the induction of IFNs (150). Activation of innate immune cells by viral genomic entities initiates IFN responses, i.e. IFNα and IFNβ. These type I IFN have direct antiviral effects on infected cells by inducing the expression of multiple IFN-stimulated genes (151). A number of these genes, including protein kinase R (PKR), Mx proteins, ISG-15, RnaseL/2,5-OAS and RNA helicases, have been well-characterised, and via different mechanisms all have potent antiviral activity (152).

TLR3, TLR7, TLR8 and TLR9 are localized in the endosomal membranes, with the ligand binding domain facing the lumen of the endosomes and the TIR signaling domain positioning in the cytoplasmic side. It has been demonstrated that TLR3, TLR7 and TLR8, and TLR9 recognize virus- or dying cell-derived double-stranded RNA, G/U-rich ssRNA, and unmethylated CpG DNA, respectively (150). The endosomal localization of TLR3, TLR7, TLR8 and TLR9 is essential for signaling, as demonstrated by an effective IFN production upon TLR induction by a formulation of nucleic acids that sustain their localization in the endosome (153). In line with this notion, plasmacytoid dendritic cells, being able to effectively retain viral RNA in the endosome, are strong type I IFN producers. In contrast, in conventional dendritic cells, viral RNA is rapidly transported from endosome to lysosome, rendering them weaker IFN producers.

In many other cell types, including conventional dendritic cells, macrophages and fibroblasts, TLRs and other cytoplasmic receptors (e.g. RIG-I, MDA-5) are available to initiate IFN production pathways (154, 155). However, genetic experiments have demonstrated the essential role of TLR7 and TLR9 in the IFN induction in plasmacytoid dendritic cells by RNA viruses (156). IFN production by plasmacytoid dendritic cells upon ligation of TLR7 is mediated by MyD88-IRAK-TRAF6.

TLR7 and TLR9 signaling activate IRF7, the master regulator of IFNα. It has been shown that MyD88 and TRAF6 can bind to IRF7 directly, and recruit IRAK1 to phosphorylate IRF7, resulting in the nuclear translocation and activation of IRF7 (157, 158). Indeed, IRAK1-deficient mice are defective in IFNα production in response to stimulation of TLR7 and TLR9 (159). These facts highlight the importance of plasmacytoid dendritic cells as a target therapy for chronic viral infection, via ligation of TLR7 and TLR9, to induce the production of antiviral IFN, to inhibit viral replication and clear the infection.

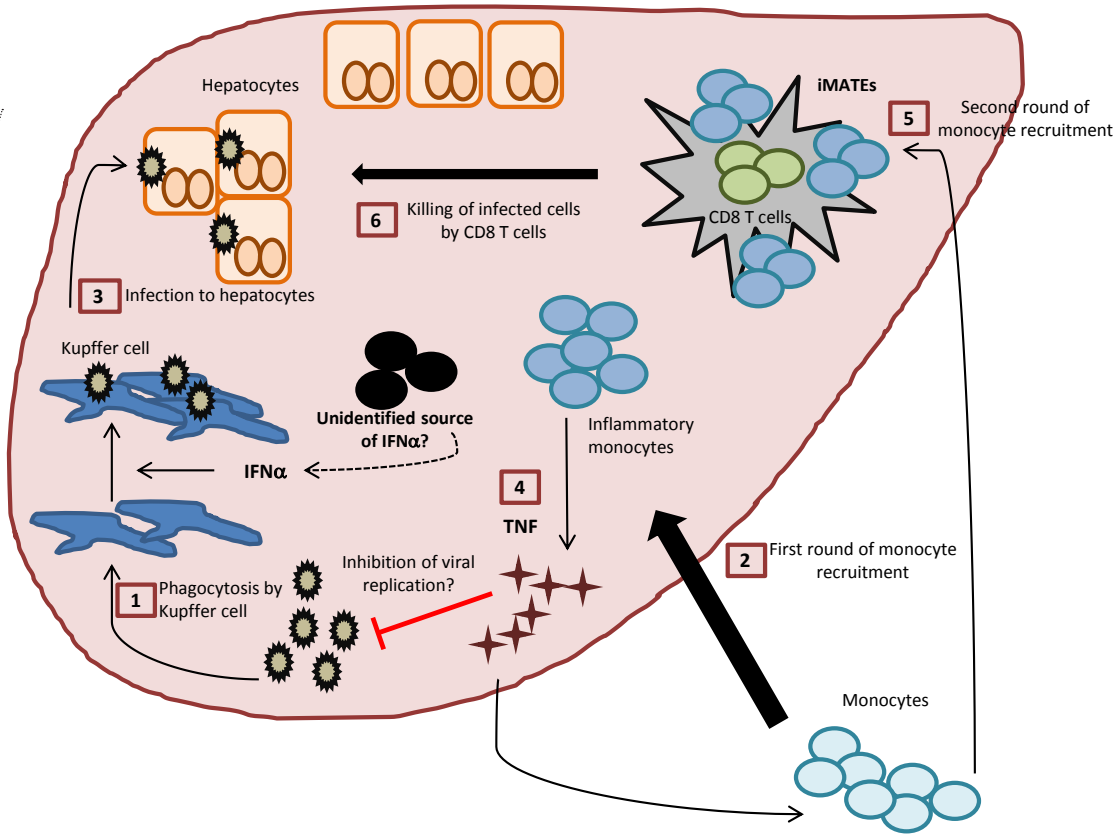

Figure 4. The proposed role of Kupffer cells and inflammatory monocytes in LCMV-induced viral hepatitis. Early after infection, LCMV are taken up by Kupffer cells (1), accompanied by the first influx of inflammatory monocytes to the liver (2). The ability of Kupffer cells to take up LCMV particles is modulated by IFNa and might limit hepatocyte infection (3). Inflammatory monocytes produces TNF (4) which mediate recruitment of more inflammatory monocytes to the liver (5) and might role in inhibiting LCMV replication. Recruited inflammatory monocytes form iMATEs (intrahepatic myeloid-cell aggregates for T cell population expansion) where $CD8^+$ T cells are propagated and participate in killing infected hepatocytes (6).

Aims and outline of the thesis

Since HBV and HCV replicate in liver parenchymal cells, understanding the modulation of intrahepatic immune responses by these viruses is essential to delineate host factors that contribute to disease pathogenesis. Kupffer cells, the liver-resident macrophages, are abundantly present in the sinusoids of the liver. Due to their location, these cells are constantly exposed to oxygen- and antigen-rich blood circulation. Currently, we have poor understanding of how these cells function under steady state conditions or in liver disease. This is mainly due to the lack of consensus in identifying Kupffer cells, as well as the different methods applied to isolate these cells. Both immune stimulatory and regulatory functions have been demonstrated for Kupffer cells. However, the functional characteristics of Kupffer cells in a steady state, or in HBV and HCV infection are still not fully understood.

In **Chapter 2, Chapter 3 and Chapter 4**, the functional and phenotypical characteristics of Kupffer cells were reviewed and investigated. Taking into account the challenges in distinguishing Kupffer cells from infiltrating monocytes/macrophages and other myeloid cells during liver inflammation, in **Chapter 2**, we discuss our current understanding of the function of Kupffer cells, and assess their role in the regulation of anti-viral immunity and disease pathogenesis during HBV and HCV infection.

At present, under steady state conditions, Kupffer cells are considered immune tolerant. In order to get more insight into the role of Kupffer cells in modulating the intrahepatic immune response, in **Chapter 3**, phenotypic and functional aspects of Kupffer cells from healthy C57BL/6 mice were analyzed and compared to those of splenic and peritoneal macrophages.

Besides their role under steady state conditions, in **Chapter 4**, the phenotype and function of Kupffer cells were studied during the early phases of viral hepatitis induced by LCMV clone 13. It is expected that viral infection in the liver poses a continuous stimulation to Kupffer cells and this might alter their functional characteristics.

In light of the unique phenotypical and functional characteristics demonstrated by Kupffer cells, we set to investigate the role of Ctcf, the DNA binding zinc-finger protein CCCTC-binding factor (Ctcf), in modulating these features. Ctcf has been shown to coordinate specific communication between transcription factors and gene expression processes. However, in contrast to the adaptive immune cells, the role of Ctcf in development and function of myeloid cells, particularly Kupffer cells, *in vivo* has not been investigated. In **Chapter 4**, we use the transgenic Cre-loxP system to generate conditional myeloid-specific *Ctcf*-knockout mice to investigate the role of Ctcf in the development and function of macrophages.

Monocytes are the precursors of tissue-resident macrophages and dendritic cells and rec-

ruited to inflamed organs upon pathogen assault. Contradictory roles of intrahepatic inflammatory monocytes during sterile toxin-induced liver inflammation have been described. Considering that a high number of inflammatory monocytes are recruited to the liver during inflammation, it is expected that they play a role in shaping the intrahepatic immune response, and thereby affecting the outcome of a viral infection in the liver. However, due to the lack of small animal models, our understanding on the role of these cells during early onset of virus-induced liver disease is limited. It is not clearly understood whether, following recruitment to the liver, inflammatory monocytes display a macrophage-like or dendritic cell-like phenotype and function, and whether they contribute to the intrahepatic microenvironment and interact with other intrahepatic immune cells to modulate their functions. In **Chapter 5**, the phenotype and function of intrahepatic inflammatory monocytes during the early phases of LCMV infection were characterized. Finally, we examined the possibilities of therapeutic manipulation using TLR7 ligation in the chronic LCMV model. TLR7 ligation is currently being investigated as an alternative therapy in chronically infected patients to directly activate immune cells and induce the production of antiviral IFNs. Considering that HBV and HCV replicate solely in the liver, a better understanding of the intrahepatic immune responses induced by TLR7 ligation is essential to evaluate the efficacy and safety of the therapy. Due to the complexity of performing immunological studies on the liver, little information is available on the intrahepatic IFN responses induced by LCMV infection alone or in combination with TLR7 treatment. In **Chapter 6**, we use LCMV clone 13 infection in mice as a model of persistent viral infection and investigate the intrahepatic events upon systemic TLR7 ligation using R848.

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2

The role of Kupffer cells in hepatitis B and hepatitis C virus infections

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Summary

Globally, over 500 million people are chronically infected with the hepatitis B virus (HBV) or hepatitis C virus (HCV). These chronic infections cause liver inflammation, and may result in fibrosis/cirrhosis or hepatocellular carcinoma. Albeit that HBV and HCV differ in various aspects, clearance, persistence and immunopathology of either infection depends on the interplay between the innate and adaptive responses in the liver. Kupffer cells, the liver-resident macrophages, are abundantly present in the sinusoids of the liver. These cells have been shown to be crucial players to maintain homeostasis, but also contribute to pathology. However, it is important to note that especially during pathology, Kupffer cells are difficult to distinguish from infiltrating monocytes/macrophages and other myeloid cells. In this review we discuss our current understanding of Kupffer cells, and assess their role in the regulation of anti-viral immunity and disease pathogenesis during HBV and HCV infection.

The characteristics of Kupffer cells

Kupffer cells (KC) are tissue-resident macrophages residing in the liver. They are located in the liver sinusoids, and are the largest population of innate immune cells in the liver (1-3). Due to their abundance and localization, KC are crucial cellular components of the intrahepatic innate immune system that are specialized to perform scavenger and phagocytic functions, thereby removing protein complexes, small particles and apoptotic cells from blood (1-3). Together with the sinusoidal endothelial cells, KC are the first barrier for pathogens to enter the liver via the portal vein (4). This is extremely important, since venous portal blood is rich in pathogen-derived products, such as lipopolysaccharide, and pathogens from the gut, which need to be eliminated from the circulation to avoid systemic immune activation.

The specialized function of KC is reflected by the phenotype: they were identified in the early 1970s as peroxidase-positive cells with cytoplasm containing numerous granules and vacuoles, and occasional tubular, vermiform invaginations (5-8). At present, human KC are identified by immunohistochemistry or flow cytometry using antibodies directed against CD68, CD14 and CD16 (9-11). However, it is important to mention that these markers are not unique for human KC and macrophages from other tissues, but are also expressed on monocytes, which are also considered a source of precursor cells for KC, and/or dendritic cells (12). Different from their human counterpart, rat KC are commonly identified by antibodies against CD68 or CD163 (ED1 and ED2, respectively) (13), and mouse KC using the F4/80 marker (14). However, also the rat and mouse markers are not unique for KC, but are shared with other leukocytes.

The ambiguity in the identification of KC that exists under steady state conditions is even more challenging under pathological conditions in which cellular infiltrates are observed consisting of inflammatory monocytes and/or dendritic cells that share certain surface markers. In rat studies, large and small KC were shown to be present in distinct area within the liver, i.e. in the peri-portal, and peri-venous and mid-zonal area, respectively (10, 15-19), and 2 subpopulations of KC have been isolated from rat liver tissue: $ED1^{+}ED2^{-}$ and $ED1^{+}ED2^{+}$ cells (16, 17). Similarly, some studies have identified 2 subpopulations of mouse KC: $F4/80^{+}CD68^{+}$ and $F4/80^{+}CD11b^{+}$ cells from mouse liver tissue (20). It is likely that these populations either illustrate distinct differentiation phases rather than distinct KC subpopulations, or that they identify infiltrating monocytes instead of resident tissue macrophages. In studies from our group, we defined only one KC population in mouse liver tissue on the basis of F4/80 and CD11b expression (21). This was in line with a study in human where only a single population of KC was identified as $CD14^{+}$, $HLA\text{-}DR^{+}$, $HLA\text{-}ABC^{+}$, $CD86^{+}$ and $DC\text{-}SIGN^{+}$ cells, with low expression of CD1b, CD40 and CD83 (9). It is preferable to identify KC not solely based on the available markers, but also on their morphology and phagocytic ability as

their hallmark function. In this review, KC are identified as $CD68^+$, $CD14^+$ and/or $CD11b^+$ cells (human), $ED1^+$ and/ or $ED2^+$ cells (rat) and $CD68^+$, $F4/80^+$ and/ or $CD11b^+$ cells (mouse), according to the original studies. Under steady state condition, the majority of tissue-resident macrophages in the mouse liver have a yolk sac origin and are self-maintained. Upon serious challenge, tissue resident KC can be replaced by precursor cells from bone marrow as well as monocytes, which develop into tissue-resident macrophages (22). Since the distinction between tissue-resident KC and tissue-infiltrating monocyte/macrophages is difficult, and since most studies did not discriminate between these cells with a different origin, we will use the term "KC" to describe both cells.

Studies on human KC are being performed using cells obtained from liver tissue or from liver graft perfusate. Liver graft perfusate is preserved in a different manner than liver tissue. Also, tissue-derived KC are commonly isolated using collagenase, a processing step not included for perfusate, which increases the amount of extracellular debris and may induce phenotypic and functional changes. The source of liver material as well as the method to process the samples are important to take into account when interpreting results on the phenotype and function of KC from the various studies.

Macrophages are specialized in sensing and responding to pathogens and equipped with specific pattern recognition receptors, including scavenger receptors, Toll-like receptors (TLR), RIG-like receptors (RLR), NOD-like receptors (NLR) and C-type lectins. These receptors are expressed by tissue-derived as well as *in vitro*-generated macrophages (reviewed in (23)). However, only few of them have been described for KC and it is not clear whether the others are expressed by KC. Scavenger receptors and C-type lectins are important receptors mediating phagocytosis, which are expressed by human, rat and mice KC (24-26). The phagocytic ability of human KC has been shown in relation to removal of erythrocytes, apoptotic cells and debris (27, 28). In line with that notion, we and others have shown that rat and mouse KC are strongly phagocytic and possess a high level of basal reactive oxygen species (ROS) production (20, 21). Upon *in vivo* administration of dextran particles, *E. coli* or gadolinium chloride, rat and mouse KC take up these particles, produce high levels of ROS, and demonstrate high lysosomal activity (17, 18, 20, 21). Human KC were shown to express TLR2, TLR3 and TLR4 (9, 29). The expression of other TLR, as well as NLR and RLR have not been described, but cannot be excluded since the murine counterparts were found to express functional TLR1-TLR9 and RIG-I (25, 30). In human and rodents, ligation of TLR on tissue-derived and *in vitro*-generated macrophages resulted in cytokine production (31). However, to date, studies on the ability of KC to produce cytokines upon TLR ligation resulted in divergent conclusions. For instance, we and others show that KC from human liver tissue and perfusate release IL-10, IL-1β, IL-6, IL-12, IL-18 and TNF upon TLR2, TLR3 and TLR4 ligation *ex vivo* (9, 32, 33) and [Boltjes, unpublished data]. Similarly, Kono et al showed that liver tissue-derived rat KC produce superoxide, TNF and IL-6 upon TLR4 ligation *ex vivo* (17).

Table 1: Surface molecules and secreted inflammatory mediators facilitating KC roles in HBV/HCV infection.

	HBV		**HCV**	
	Mediators	**Reference**	**Mediators**	**Reference**
Binding/ Uptake				
	HSPG	79	HSPG	79
	CD14	9	SR-B1	82
	mannose receptor	69	LDL-receptor	81
			DC-SIGN	9, 85
Pattern Recognition Receptors				
			TLR2	84, 87
			TLR4	88
Cytokines				
	IL-1β	89	IL-1β	84, 100
	IL-6	89	TNF	84
	TNF	89	IL-10	84
	TGFβ	91		
Chemokines				
	CXCL8	89		
Co-stimulatory molecules				
			CD40	94
			CD80	94
			MHC class II	94
Immune inhibition or promotion of tolerance				
	TGFβ	91	PD-L1	84, 137
	PD-L2	30	IL-10	84
	galectin-9	135	galectin-9	120
Liver damage				
	IL-6	89	TRAIL	84
	TRAIL	84	granzyme B	105, 106
	FasL	106	perforin	105, 106
	granzyme B	105		
	perforin	105		
	ROS	54		
	galectin-9	135		
	TGFβ	91		

However, examination of mouse KC isolated from liver tissue by our group and others demonstrated weak induction of TNF and IL-12p40 upon *ex vivo* stimulation with agonist for TLR4, TLR7/8 or TLR9 (20, 21), whereas no data are available on the cytokine-producing ability of liver perfusate-derived rat or murine KC. Thus, more studies using highly purified KC with a well-defined phenotype need to be conducted to obtain conclusive data on the TLR responsiveness of KC.

A weak ability of KC to produce cytokines might be related to their tolerogenic function in a steady state condition. KC are frequently exposed to gut-derived antigens. Instead of exerting inflammatory responses, human and murine KC constitutively express TGF-β and PD-1, possess high levels of negative regulators downstream the TLR pathway and secrete IL-10 upon LPS stimulation (20, 21, 32, 34-36). More importantly, the ability of murine KC to produce pro-inflammatory cy-

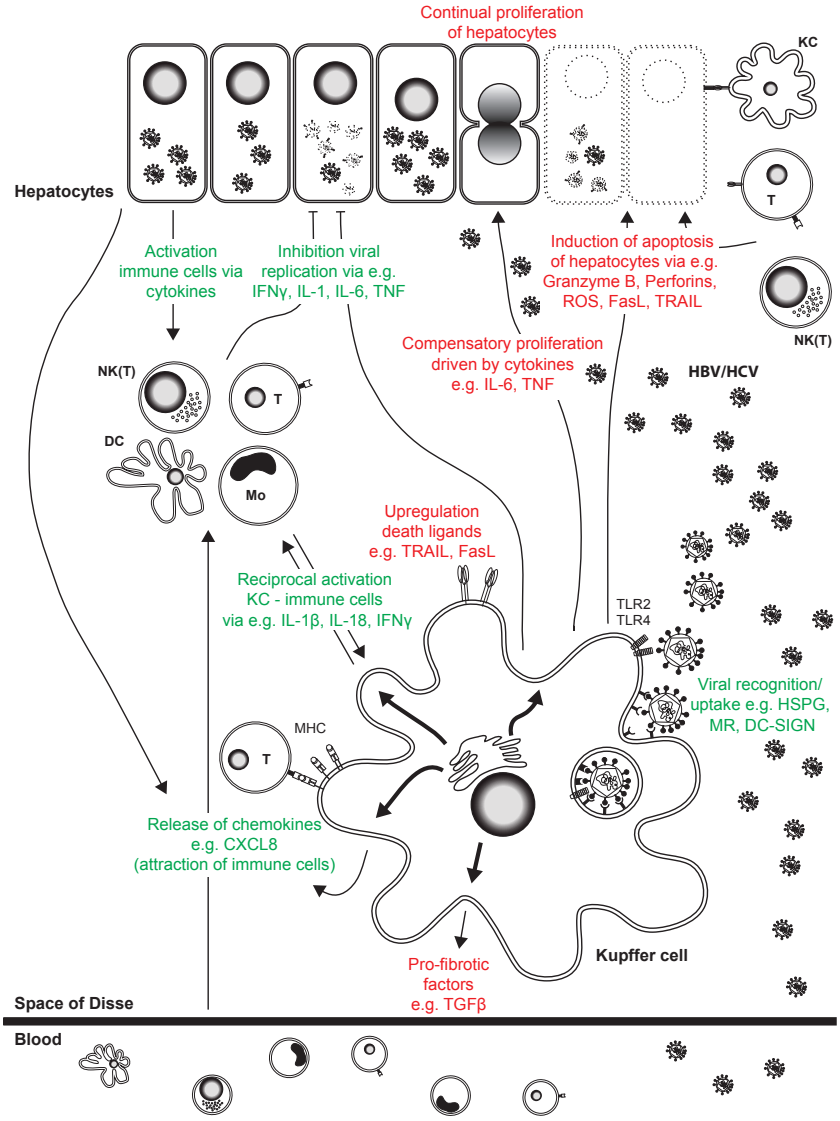

Figure. 1. The role of KC in anti-viral immunity and tissue damage during HBV and HCV infection. Exposure of KC to HBV or HCV will lead to direct activation of KC that, together with infected hepatocytes, release cytokines and chemokines, which are responsible for the attraction of other leukocytes. Activation of infiltrating immune cells leads to further production of cytokines that indirectly activate KC. The secreted cytokines may inhibit viral replication (**green text**). However, persistent exposure of KC to HBV or HCV will continuously activate KC leading to the ongoing release of cytokines and chemokines attracting and activating more leukocytes. Likewise, continuous activation of infiltrating leukocytes leads to ongoing production of cytokines that indirectly activate KC. Some of the cytokines secreted are pro-fibrotic factors.
Additionally, KC and other immune cells are able to induce apoptosis of infected as well as uninfected hepatocytes, and release cytokines, which drive compensatory proliferation of hepatocytes. The ongoing cycles of hepatocyte death and regeneration increase the chances of spontaneous mutations and DNA damage, which may eventually result in HCC (**red text**).

tokines upon TLR4, TLR7/8 and TLR9 is by far weaker than that of peritoneal macrophages (21). This observation suggests that KC play a crucial role in maintaining liver homeostasis in a steady state condition. Additionally, our mouse study and others show that KC are superior in the ability to take up particles and have a higher basal ROS production, in comparison to splenic and peritoneal macrophages, which highlight their function to remove particulates from the circulation (21, 37).

The role of KC during LCMV infections

Besides their barrier (4) and janitor function (38, 39), KC have been shown to play a role in the response to pathogens, including viruses. Studies on the importance and anti-viral immune functions of KC in HBV and HCV infections are difficult to perform, since these viruses only infect and replicate in humans and non-human primates, and immunocompetent small animal models for viral hepatitis are not yet available (reviewed in (40, 41)). As an alternative approach several mouse infection models, including lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), mouse hepatitis virus (MHV) and adenovirus models, have provided information on the role of KC in viral infection. However, in contrast to HBV and HCV where infection and replication is restricted to hepatocytes, these hepatitis mouse models also infect other cells and even other organs. Of these models, MHV and LCMV have been shown to replicate in KC (42, 43). LCMV, MHV and adenovirus particles can be taken up from the circulation by murine KC via scavenger and complement receptors, which may limit infection (44-47). It has been shown that failure in clearing LCMV, MHV and adenovirus particles during the acute phase results in "spill-over" infection of hepatocytes, prolonged infection and exacerbated immunopathology (47-49). Studies using these mouse models have been instrumental in our understanding of the effects on KC during the early phases of virus infections. A number of studies have also evaluated KC during persistent infection in mice. These studies are conducted using specific isolates of LCMV, the clone 13 and WE strains. The development of persistent infection with a high rate of replication of LCMV is similar to HBV and HCV, and important mechanistic pathways identified in LCMV infected mice, were later confirmed to be operational during chronic viral infections in patients. However, in contrast to HBV and HCV, murine LCMV infections are not restricted to the liver, and LCMV replication can also be found in the spleen, lung and kidney. The long-term consequences of human viral hepatitis, such as fibrosis, are absent in mice, although virus-induced liver damage is observed (44, 50). The effect of chronic LCMV infection on NK cells and virus-specific T cells has been extensively examined, however only few studies have focussed on KC. In contrast to HBV or HCV, active replication of LCMV in the liver, as evidenced by the detection of viral RNA and antigen, has been demonstrated in KC as well as in hepatocytes (43, 51, 52). During the first 2 weeks following LCMV infection, an increase of the number of $F4/80^+$ cells is ob-

served, followed by normalization of their numbers (19). Although differences in MHC class-I expression levels were observed within the F4/80 population by immunohistochemistry, the relative contribution of infiltrating monocytes versus enhanced activation of resident KC is difficult to determine.

An elegant study by Lang et al. showed that clodronate-mediated depletion of KC resulted in rapid LCMV dissemination due to the inability to capture virus, which led to replication within hepatocytes and subsequently severe $CD8^+$ T cell-mediated liver damage (44). The study further showed that KC responded to type I IFN by inducing the expression of interferon-stimulated genes, and that mice lacking IFNAR specifically on macrophages exhibited strongly enhanced viral titers. However, recently a detrimental influence of granulocytes and macrophages in spleen and liver was reported by their ability to produce reactive oxygen species (ROS) following viral infection, although ROS production by liver $F4/80^+$ cells was low (53). Importantly, the effect of ROS was an impairment of the immune response, and in the absence of ROS mice exhibited lower viral titers and less liver damage. In a different experimental mouse model, which makes use of transgenic intrahepatic expression of the HBV large envelope protein, ROS activity was observed in KC, and these mice exhibited a chronic necroinflammatory liver disease, resembling human chronic active hepatitis (54).

The findings from the LCMV mouse model clearly show the complexity of the anti-viral response in the liver since KC can both contribute to promote and suppress viral eradication and liver pathology. In the following section, we will focus on the interaction of KC with HBV and HCV, and the functional consequences.

The role of KC during HBV and HCV infections

Both HBV and HCV are transmitted predominantly via percutaneous and sexual exposure, while perinatal exposure is often seen for HBV only (55-57). Infection with these viruses can either resolve spontaneously or develop into chronic liver disease with continuous viral replication in hepatocytes (56-58). Chronic hepatitis poses an increased risk for liver fibrosis and cirrhosis, hepatic failure, and hepatocellular carcinoma (HCC) (58, 59). Patients with a self-limiting HBV or HCV infection show sustained, vigorous and multi-epitope-specific $CD4^+$ or $CD8^+$ T cell and B cell responses, whereas in chronic HBV and HCV these responses are weak and/or transient (60-63). This demonstrates that clearance of the infection is dependent on strong multi-epitope-specific T and B cell responses, which is only possible following effective innate immune responses (63, 64). Here, we will firstly address the role of KC in the interaction and recognition of HBV and HCV, and their role in the induction of a pro-inflammatory response. Pro-inflammatory mediators are important for inhibition of viral replication, the induction of resistance to infection of neighboring cells, and attraction and activation of other immune cells, and consequently contribute to the development of

effective virus-specific immunity. Secondly, we will discuss KC-virus interactions that may inhibit the development of effective viral immunity, facilitate viral persistence or promote liver damage.

Interaction of KC with HBV and HCV

HBV is a 3.2 kb partially double-stranded DNA envelope-virus which replicates via RNA intermediates. Hepatitis B core protein (HBcAg)-encapsulated viral DNA and hepatitis B envelope protein (HBsAg) form a complete viral or Dane particle. HBV particles, HBsAg, and hepatitis B early antigen (HBeAg; a truncated form of HBcAg) are secreted by infected hepatocytes and can be detected in serum of HBV patients (58, 65).

Evidence for productive HBV infection of cells other than hepatocytes is lacking. Also, detailed information on the presence of HBV (proteins) in KC *in vivo* or the uptake of HBV or its proteins by human KC *ex vivo* has not been reported. Although no information is available on KC, studies using THP-1 monocytic cells, monocytes and dendritic cells have shown binding of HBV or HBV proteins, leading to their activation. For instance, TLR2 and heparan sulfate proteoglycan (HSPG) were suggested to be responsible for HBcAg recognition on THP-1 cells, and HBcAg-induced activation of THP-1 cells resulted in production of IL-6, IL-12p40, and TNF (66). However, since HBcAg is only found within infected hepatocytes or viral particles, it is unclear whether HBcAg interacts with KC, via HSPG and/or another extracellular receptor like TLR2. Also, other receptors expressed by KC are known to interact with HBV proteins as demonstrated in other cell-systems (Table 1). For instance, HBsAg can interact with human blood monocytes in a CD14-dependent fashion (67), and with dendritic cells via the mannose receptor (68), which are both receptors known to be also expressed on KC (69). Finally, complex formation of HBsAg with albumin may lead to enhanced uptake of HBsAg from the circulation by KC and endothelial cells (70).

HCV contains a 9.6 kb positive-strand RNA genome that translates into the structural proteins, core and E1 and E2 envelope proteins, and the non-structural proteins NS1-NS5. After replication, they form a small-enveloped virus particle containing the newly synthesized RNA genome (71, 72).

Compared to HBV, there is a better understanding of the entry receptors on hepatocytes used by HCV. In addition to claudin1, occludin, epidermal growth factor receptor (EGFR) and ephrin type-A receptor-2, HCV infects hepatocytes by attaching to HSPG, low-density lipoprotein (LDL) receptor, scavenger receptor (SR)-B1 and CD81. Some, but not all, receptors are expressed by KC (Table 1) (73-82). It has been reported that incubation of human liver cells with HCV-E2 resulted in HCV-E2 binding to KC in a CD81-dependent manner (83), but also DC-SIGN, a C-type lectin not expressed by hepatocytes, has been demonstrated to bind HCV on KC (84-86).

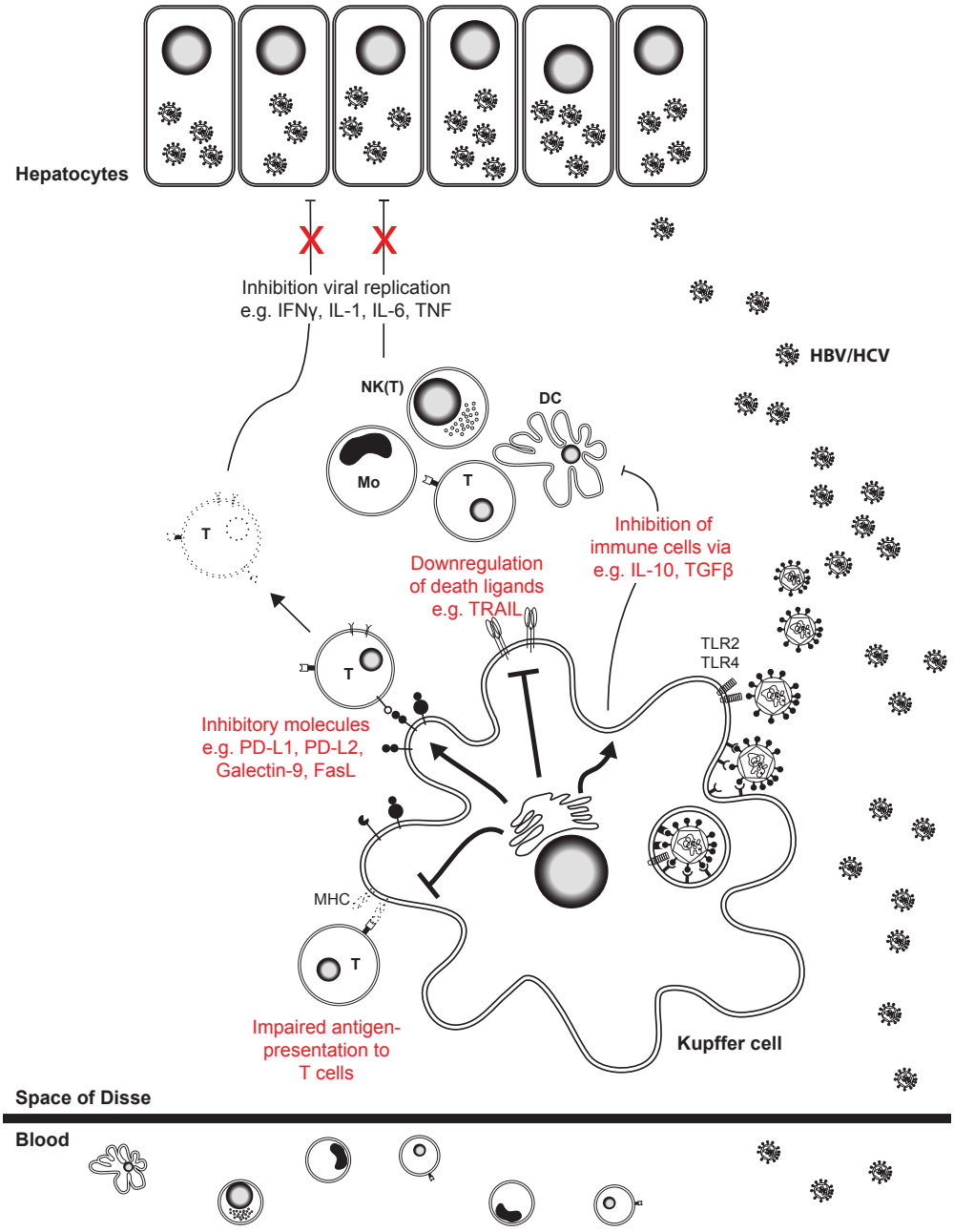

Figure 2. Role of KC in immune regulation and viral persistence during HBV and HCV infection. Exposure of KC to HBV or HCV will lead to their activation and the release of anti-inflammatory cytokines and expression of inhibitory molecules. Combined with impaired antigen presentation by KC, these regulatory mechanisms will interfere with KC function and that of other immune cells, frustrating anti-viral immunity.

Although it is unlikely that HCV can replicate in KC, activation of KC by HCV and its proteins has been demonstrated. HCV core and NS3 stimulate human liver perfusate-derived $CD14^+$ KC and monocyte-derived macrophages via TLR2 to produce pro-inflammatory IL-1β, IL-6, and TNF and immunosuppressive IL-10 (84, 87). Recently, it was shown that TLR4, in density gradient- and adherence-isolated liver-derived human KC, mediates NS3 recognition, resulting in TNF production (88). However, HCV core and NS3 are not secreted at significant levels by infected hepatocytes, posing little relevance to extracellular recognition of HCV by KC via these TLR. Alternatively, phagocytosis of infected hepatocytes by KC may allow intracellular exposure to viral RNA, but so far no evidence exists.

Stimulatory effects of HBV or HCV on KC function

There are only few publications that show a stimulatory effect of HBV or HBV proteins on the function of KC. Hösel et al. showed that HBV particles and HBsAg induce IL-1β, IL-6, CXCL8 and TNF production by human $CD68^+$ cell-enriched non-parenchymal cells via NF-κB activation (89) and subsequently inhibit HBV replication in primary hepatocytes. This inhibitory effect was mainly ascribed to IL-6, but also TNF inhibited HBV replication in a non-cytopathic manner (90). In contrast, Li et al. demonstrated that rat $ED1^+$ adherent KC exposed to HBV virions hardly expressed IL-1β, IL-6 or TNF, but produced the immunoregulatory cytokine TGFβ (91).

During chronic HCV infection, KC are increased in numbers in the liver (92, 93), and exhibit an activated phenotype with higher mRNA expression levels of the activation markers CD163 and CD33 in livers of chronic HCV patients versus controls (94, 95). Recently, it was reported that in response to HCV human KC release IL-1β and IL-18 *in vitro* (96). In line with these findings, stimulation of $CD14^+CD68^+$ cells from liver perfusate with UV irradiated cell culture-derived HCV induced IL-1β production. To support this data, *in vivo* co-expression of IL-1β and CD68 was observed using immunofluorescence on liver tissues from patients with chronic HCV (97). Besides intrahepatic IL-1β, also elevated serum IL-1β levels were detected in patients as compared to healthy individuals (97).

Although a direct effect of HCV-exposed KC on HCV replication is unknown, it was recently reported that KC-derived TNF increased the permissivity of hepatoma cells to HCV. In this study, LPS as well as HCV induced KC to produce TNF, thereby indirectly promoting HCV infection (33). On the other hand, HCV- or TLR-ligand-induced KC-derived cytokines, such as IL-6, IL-1β, and IFNβ (84, 87, 97, 98), were found to inhibit HCV replication in the HCV replicon model (98-100), implying that KC are also capable of displaying antiviral activity upon HCV exposure.

In addition, release of chemokines and cytokines by KC has an indirect effect on the immune response in the liver by recruitment and activation of infiltrating leukocytes, as also discussed by

Heydtmann et al. (101). This may result in a complex interaction between factors produced by liver parenchymal cells, liver resident immune cells including KC, and infiltrating leukocytes. KC are able to activate NK cells and NKT cells, both present at relatively high numbers in the liver, via the production of pro-inflammatory cytokines (9). In turn, NK and NKT cells produce cytokines such as TNF and IFNγ and are cytotoxic in nature (9, 102). Upon HBV exposure, KC were found to produce CXCL8 (89), which potentially attracts NK and NKT cells during the early phase of HBV infection. KC are also able to recruit dendritic cells to the liver, which involved C-type lectins interactions (103). This enhanced dendritic cell recruitment may initiate and promote virus-specific T cell responses. In contrast to dendritic cells, KC are less efficient in priming naïve T cells. Nevertheless, mouse KC have been shown to present antigen to CD4+ and CD8+ T cells, inducing these to proliferate and produce IFNγ (104, 105). The relatively high expression of CD40, CD80 and MHC class II found on CD68+ cells in chronic HCV patients (94) might point towards possible antigen presentation by intrahepatic macrophages.

Although lymphocytes such as NK cells and $CD8^+$ T cells are potent effector cells responsible to kill virus infected cells, KC have been reported to express cytotoxic molecules such as TRAIL, Fas-ligand, granzyme B, perforin and ROS, enabling them to lyse infected hepatocytes (106-108). However, since KC act in an antigen-nonspecific manner and hence can lyse hepatocytes irrespective of their infection state, it is tempting to speculate that KC cause more damage to the organ due to their cytotoxic capacity than that they provide protective immunity to the host.

In summary, only limited information exists on the direct interaction between HBV and HCV with KC *in vivo* and *ex vivo*. Macrophages are able to bind HBV or HCV or virus-related proteins *in vitro,* triggering surface and/or intracellular receptors. However, receptors used for these purposes need to be further investigated. Several studies indicate that KC may play a role in controlling HBV and HCV infections by inhibiting viral replication, either directly via the production of cytokines or via their interaction with other cells, as well as in shaping the inflammatory response towards the induction of virus-specific immunity. However, more research is required to get a better insight into the role of KC in regulating intrahepatic immunity.

Suppressive effects of HBV and HCV on KC function

Besides the contribution of KC to viral clearance, viruses may actively interfere with the pro-inflammatory functions of KC to evade host immunity. Various studies show that HBV and HCV are able to interfere with TLR pathways, RIG-I signaling and subsequent pro-inflammatory activities of hepatocytes and immune cells (109-113), but studies describing the effect on human KC are limited. Only one study described that type I IFN production and TRAIL expression by human perfusate-derived KC were suppressed by HCV core protein via disruption of the TLR3/TRIF/TRK1/IRF3 pathway (84). In addition, nu-

merous studies on monocytes have demonstrated modulation of cytokine production by HCV proteins, and altered TLR responsiveness of monocytes obtained from chronic HCV patients (114-116).

Concerning HBV, pretreatment of non-parenchymal cells including KC, with HBV-Met cell-derived supernatants, HBsAg, HBeAg or hepatitis B virions almost completely abrogated TLR-induced anti-viral activity, i.e. IFNβ production, interferon-stimulated gene (ISG) induction, IRF3, NF-κB, and ERK1/2 expression (117). Accordingly, incubating human monocytes with HBeAg or HBsAg inhibited TLR2-induced phosphorylation of p38 MAPK and JNK MAPK, and subsequent production of IL-6, TNF, and IL-12 (29, 118, 119). *In vivo,* TLR2 expression by KC and peripheral blood monocytes in HBeAg-positive chronic HBV-infected individuals was lower than that in HBeAg-negative patients and controls. Moreover, TLR2 ligation induced less IL-6 and TNF in those HBeAg-positive patients (29). These alterations may be related to the inhibitory effect of HBeAg on TLR2 signaling demonstrated *in vitro*. In addition, also TLR3 expression was found to be lower on PBMC from chronic HBV patients compared to control patients as well as on liver cells, including KC (120). Antiviral therapy of chronic HBV patients with entecavir or pegylated IFN-α partially restored TLR3 expression, but it is unclear whether this is a direct viral effect.

Tolerogenic effects of HBV and HCV related to KC

As mentioned above, KC are constantly exposed to pathogen-derived products from the gut. To prevent excessive inflammation and pathology of the liver, continuous activation of KC is avoided as these cells become refractory to subsequent endotoxin challenge, a phenomenon known as endotoxin-tolerance (121, 122). This contributes to the well-described tolerogenic milieu in the liver. Besides modulation of TLR-signaling pathways, also expression of anti-inflammatory mediators, such as IL-10 and TGFβ, and other soluble and membrane-bound inhibitory molecules are underlying the intrahepatic tolerance (35, 105, 122, 123).

A number of studies have reported that HBV and HCV components affect the production of immunoregulatory cytokines, and consequently promote the tolerogenic milieu of the liver. In this respect, it has been reported that HBV particles preferably induced TGFβ production by rat KC instead of pro-inflammatory cytokines (91). One of the activities of TGFβ is that it plays a role in maintaining tolerance towards self-antigens by selectively supporting the differentiation of $FoxP3^+$ regulatory T cells (124, 125). Furthermore, HCV core protein induces IL-10 production by human KC (84, 87). Elevated intrahepatic IL-10 levels may suppress pro-inflammatory cytokine production by intrahepatic cells, frustrate KC-NK cell interaction (9, 126) and antigen presentation to T cells and their activation (105, 127-133). Interestingly, chronic HBV and HCV patients showed higher plasma levels of IL-10 than uninfected individuals (134, 135), which could be the result of a direct viral effect

on KC and/or other cells, or the result of a negative feedback mechanism resulting from ongoing liver inflammation. Recently, the role of KC was examined in an established HBV-carrier mouse model. In this model, KC as well as IL-10 were involved in the establishment of antigen-specific tolerance towards peripheral HBsAg vaccination (136).

KC express membrane-bound inhibitory ligands that could facilitate a tolerogenic milieu in the liver. For instance, under steady state conditions, KC are known to express PD-L1, which is a ligand for PD-1 and known to impede T cell function by inhibiting proliferation and cell division (36). Immunohistochemical analyses of liver biopsies from chronic viral hepatitis patients revealed that $CD68^+$ macrophages expressed increased levels of PD-L2 compared to control liver tissue (30, 123, 137). Similar results were reported for galectin-9 with enhanced expression by $CD68^+$ cells by immunohistochemistry, which was confirmed by flow cytometry (137). Interestingly, enhanced serum levels of galectin-9 were observed in patients with biochemical evidence of highly active chronic HBV-related liver disease (ALT>100 U/L) as compared to patients with relatively low ALT levels (<50 IU/L) or healthy controls. Also comparison of plasma galectin-9 levels in patients with chronic HCV showed higher levels in patients compared to healthy individuals (123), Furthermore, co-localization of CD68 and galectin-9 was observed in the peri-portal regions of the livers of virtually all the patients with HCV infection, regardless of grade of inflammation or stage of fibrosis, but not in normal control livers (123).

These inhibitory ligands are known to inhibit T cell function upon cell-cell contact via interaction with PD-1 and Tim-3, respectively (138), which is of relevance since both PD-1 and Tim-3 are reported to be upregulated on HBV- and HCV-specific intrahepatic and peripheral blood-derived $CD8^+$ T cells and associated with T cell dysfunction and exhaustion during chronic viral hepatitis (123, 139, 140). Intrahepatic expression levels of PD-L1, PD-L2 and PD-1 correlated with liver inflammation in chronic HBV (30). Although it has been shown that HCV core protein can induce PD-L1 expression on human perfusate-derived KC (84), it is not clear whether the upregulation of inhibitory ligands on intrahepatic macrophages and its correlation with inflammation are direct effects of HBV or HCV, or are components of negative feedback mechanisms that develop as a consequence of persistent inflammation.

Thus, several studies indicate that both HBV and HCV compromise anti-viral immunity to a certain extent by (1) interfering with signaling of pathogen recognition receptors and the production of pro-inflammatory cytokines by KC and (2) increasing the tolerogenic capacities of KC resulting in the elevated expression of anti-inflammatory mediators. As persistent inflammation in general is accompanied by negative feedback mechanisms, the KC-related anti-inflammatory signals observed during chronic viral hepatitis could be explained by direct viral effects, immune

regulation as part of the ongoing inflammatory response, or a combination. However, also immune activating functions of KC have been described upon HBV/HCV interaction. These seemingly contradictory functions probably indicate a critical balance influenced by the extent to which receptors are triggered (or over-triggered) and also by the type of KC receptors that are triggered. Therefore, not only the concentration of virus (proteins), but also the time since infection may strongly affect KC function. Whether also age influences KC function as one of the mechanisms explaining the self-limiting hepatitis often seen in HBV-infected adults, whereas young children usually develop chronic infection, has to be investigated.

Role of KC in viral hepatitis-related liver damage

Liver fibrosis

One of the consequences of sustained low-grade injury induced by persistence of HBV and HCV in the liver is fibrosis, which is characterized by excess collagen deposition and accumulation of extracellular matrix. HBV and HCV may induce fibrinogenesis by activating hepatic stellate cells directly or indirectly by inducing cellular injury, apoptosis and necrosis, which triggers a wound healing response. KC are thought to be involved in fibrogenesis by the release of various pro-fibrinogenic factors, such as ROS and certain cytokines, such as IL-6, TNF, IL-1, PDGF and TGFβ, that induce activation of hepatic stellate cells (141). In addition, KC produce enzymes that are important or the breakdown of matrix, such as collagenases and metalloproteinases, but they also regulate the production of these factors by other cells, leading to disturbance of the homeostatic mechanisms involved in extracellular matrix deposition (142). Recent studies in experimental animal models demonstrate that these activities are only partially conducted by liver-resident macrophages, but largely depend on recruitment of monocytes as precursors of macrophages into the inflamed and damaged liver (143, 144).

Although, in patients with viral hepatitis, no causative role has been demonstrated for KC in the development of liver fibrosis, increased numbers of $CD14^{+}CD68^{+}$ KC were found around the regions of damage and fibrosis (134). These increased numbers were associated with liver injury (93, 141, 145, 146). A detailed study by Liaskou et al. observed that in liver tissue from non-viral hepatitis patients with end-stage liver disease a specific monocyte subpopulation accumulated in the liver, which was able to conduct phagocytic activity and to release inflammatory and profibrinogenic cytokines (147). Interestingly, a study in HBV replication-competent transgenic mice showed an opposite effect of KC by demonstrating that they did not contribute to liver damage, but prevented liver injury by removal of apoptotic hepatocytes during viral hepatitis (39). In this model, clodronate-mediated depletion of KC resulted in higher numbers of necrotic hepatocytes and elevated serum ALT

levels. In line with this, in a different mouse model, liver-infiltrating monocyte/macrophages mediated regression of fibrosis via phagocytosis of cellular debris (148).

Liver damage and ultimately the induction of fibrosis may be, at least in part, attributed to cytokines produced by KC. Moreover, during viral hepatitis KC have also been found to express cytotoxic molecules, like TRAIL, Fas-ligand, granzyme B, perforin and ROS, that enable them to kill infected as well as non-infected "bystander" hepatocytes (106-108). Fas-ligand expression by KC was increased in chronic HBV patients and associated with elevated ALT levels, while granzyme B and perforin expression by KC was increased in both chronic HBV and HCV patients (106, 107). Interestingly, a direct contribution of KC to the pathogenesis of hepatitis has also been reported for viral infections by viruses that infect other organs and are not detected in the liver itself (149). In influenza infection, KC were indicated as the effector cells killing hepatocytes in an as yet unidentified manner, leading to damage-associated hepatitis. KC can kill hepatocytes either directly via Fas-dependent apoptotic pathways or indirectly by interacting with CD8+ (and possibly CD4+) T cells through stimulation of cytokine secretion and other mediators, such as ROS (149).

Hepatocellular carcinoma

Chronic HBV/HCV and cirrhosis are major risk factors for the development of hepatocellular carcinoma (150). Although HCC development has been extensively studied in mice and rat, only few studies have directly assessed the importance of KC in HCC development in chronic HBV setting, and no studies are available from chronic HCV setting. Dying hepatocytes, likely resulting from anti-viral activities since HBV and HCV are considered non-cytopathic, will activate neighboring cells, including KC (151), to produce cytokines and growth factors, such as hepatocyte growth factor, IL-6 and TNF, which will further amplify the inflammatory response and drive the compensatory proliferation of surviving hepatocytes (152). Ongoing cycles of hepatocyte death and regeneration increase the chances of spontaneous mutations and DNA damage (153) eventually resulting in HCC. In HBV-transgenic mice, KC and/or infiltrating macrophages produced high levels of ROS, resulting in extensive oxidative DNA damage in neighboring proliferating hepatocytes and development of HCC (54). HBV/HCV also activate KC to produce these types of pro-inflammatory mediators, which may support the development of HCC (84, 89). Additionally, the immunoregulatory mediators expressed by KC, either as a direct virus-KC interaction or as a consequence of the inflammatory response, may also inhibit tumor-specific immune responses. For instance, galectin-9 expressed on intrahepatic macrophages caused senescence of $CD4^+$ and $CD8^+$ $Tim3^+$ T cells, and may explain part of the mechanism leading to the development of HCC (154). Furthermore, one of the HBV-derived proteins, HBxAg, also has direct tumorigenic effects (155). Hepatocyte regeneration, either influ-

enced by KC or not, allows HBxAg integration in DNA of hepatocytes, which is one of the processes involved in the development of HCC (reviewed in (153)). Whether HBxAg directly interacts with KC is not described.

In conclusion, KC play a central role in liver damage during hepatitis, having all the tools to induce inflammation, cell death, fibrosis and ultimately HCC, but further research during HBV/HCV infection remains to be carried out to determine the exact contribution of KC to liver damage in viral hepatitis.

Perspectives

Currently, our understanding of the role of KC in viral hepatitis is incomplete. The detailed contributions of liver-resident KC versus liver-infiltrating macrophages to various processes of disease pathogenesis are difficult to determine, because of the highly overlapping characteristics of these cells. Nevertheless, we can appreciate several possible anti-viral roles of KC, including binding and/or uptake of virus leading to immune recognition and the production of pro-inflammatory mediators resulting in (1) inhibition of viral replication in hepatocytes, (2) activation of neighboring cells, and (3) attraction, activation and interaction with other immune cells, which will further increase the anti-viral and inflammatory response (**Fig. 1.**). These immune activating roles of KC are beneficial to combat HBV and HCV in the early phases after infection, but may also contribute to tissue damage and the development of fibrosis, cirrhosis and HCC during chronic viral hepatitis (**Fig. 1.**). Furthermore, also immune regulatory functions of KC have been described, either as a consequence of direct virus-KC interaction, or as part of the complex tolerogenic liver environment and the ongoing inflammatory response upon HBV and HCV-infection, which may counteract the development of effective anti-viral immunity and support viral persistence and related disease pathogenesis (**Fig. 2.**).

With our growing appreciation of the roles of intrahepatic macrophages in both protective and harmful responses, intrahepatic macrophages form an interesting but complex cellular target for treatment options in viral hepatitis. The versatile features assigned to KC may partly belong to infiltrating monocytes/macrophages and therefore future efforts should focus on identifying phenotypical and/or functional characteristics discriminating KC from infiltrating macrophages. Furthermore, the function of KC and other intrahepatic macrophages will largely depend on the type, the level and duration of receptors triggered pushing the balance towards either protective or harmful responses. Identification of receptors and underlying molecular mechanisms involved in virus-cell interactions and insight into mechanisms involved in wanted and unwanted responses of the different macrophage populations that exert distinctive functions during the early and later phases of HBV/HCV infection are needed to move the field forward.

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3

Kupffer cells express a unique combination of phenotypic and functional characteristics compared to splenic and peritoneal macrophages

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Abstract

The immunostimulatory role of Kupffer cells in various inflammatory liver diseases is still not fully understood. In this study, phenotypic and functional aspects of Kupffer cells from healthy C57BL/6 mice were analyzed and compared to those of splenic and peritoneal macrophages to generate a blueprint of the cells under steady state conditions. In the mouse liver only one population of Kupffer cells was identified as $F4/80^{high}CD11b^{low}$ cells. We observed that freshy isolated Kupffer cells are endocytic and show a relatively high basal ROS content. Interestingly, despite expression of TLR mRNA on Kupffer cells, ligation of TLR4, TLR7/8 and TLR9 resulted in a weak induction of IL-10, low or undetectable levels of IL-12p40 and TNF, and up-regulation of CD40 on the surface. Kupffer cells and splenic macrophages show functional similarities, in comparison to peritoneal macrophages, as reflected by comparable levels of TLR4, TLR7/8 and TLR9 mRNA, and low or undetectable levels of TNF and IL-12p40 produced upon TLR ligation. The unique functional characteristics of Kupffer cells, demonstrated in this study, suggest that Kupffer cells under steady state conditions are specialized as phagocytes to clear and degrade particulates, and only play a limited immunoregulatory role via the release of soluble mediators.

Introduction

Kupffer cells are sessile, tissue-resident macrophages that reside in the sinusoids of the liver, and represent one of the largest macrophage populations in the body (1). Functionally, Kupffer cells are well known for their scavenging ability, thereby removing protein complexes, small particles and apoptotic cells from the circulation (1, 2).

The constant exposure of liver cells to various gut-derived commensal microbes and food-borne antigens from the portal circulation (3-5) has led to the concept that the liver is immunologically tolerant under steady state conditions in the absence of pathogenic challenge. To initiate and maintain these tolerant responses, Kupffer cells are considered to play an important role by creating an immunosuppressive local environment in the liver. Under steady state conditions, the spontaneous or induced production by Kupffer cells of IL-10, TGF-β, heme-oxygenase-1, and prostaglandins have been suggested to suppress immunity (6-9). Macrophage populations obtained from other tissue sites are thought to be less tolerogenic when compared to Kupffer cells. In addition to their scavenger function, macrophages generated *in vitro* from mouse bone marrow or obtained from the peritoneal cavity have been shown to be highly responsive to pathogens and pathogen-derived products, such as the ligands for the Toll-like receptors (TLR) (10). However, in apparent contrast to their putative tolerogenic features, Kupffer cells express distinct TLR (11, 12), and upon TLR triggering Kupffer cells have been reported to produce pro-inflammatory mediators. In this, TLR4 triggering by LPS stimulation of Kupffer cells results in production of TNF, IL-1β, IL-6, IL-12, IL-18 (12-14), and the anti-inflammatory cytokine IL-10 (6). Besides the importance of TLR4 in activating Kupffer cells, it was recently reported that Kupffer cells express TLR1-9, and are highly responsive to all TLR ligands by inducing the production of pro-inflammatory cytokines (12, 14). These findings therefore indicate that Kupffer cells are not tolerogenic, but instead highly immunoactive cells, capable to elicit potent pro-inflammatory responses. In these studies, the functionality of murine Kupffer cells was compared to liver sinusoidal endothelial cells (LSEC) and myeloid dendritic cells (DC) (12), or among distinct murine Kupffer cell subpopulations (14). No comparison of Kupffer cells with other primary macrophages derived from other anatomical sites was made, which allows drawing conclusions on the putative functional specialization of Kupffer cells.

A complicating factor in comparing different results reported in the literature is the technical difficulty to isolate and identify murine Kupffer cells from liver. Different isolation methods, including cell adherence, density gradient centrifugation, centrifugal elutriation, and cell sorting have been used to enrich or purify Kupffer cells (9, 12, 13, 15). Moreover, Kupffer cell identification is based on the expression of F4/80 (16), combined in some studies with CD11b and/or CD68 (14, 17). However, these markers are not exclusively expressed on Kupffer cells but also on other myeloid

cells, such as DC and polymorphonuclear cells (17).

In order to get more insight into the role of Kupffer cells in modulating the intrahepatic immune response, we started out performing a detailed phenotypical and functional analysis of murine Kupffer cells under steady state conditions. We systematically compared the functions of Kupffer cells and splenic macrophages, both important filters of circulating blood, and resident peritoneal macrophages. In contrast to peritoneal macrophages, highly purified Kupffer cell populations are weak producers of cytokines upon TLR ligation. However, liver macrophages exert potent endocytic activity, and display relatively high basal levels of ROS compared to splenic and peritoneal macrophages. These findings demonstrate that the function of murine Kupffer cells is highly specialized, and distinct from that of macrophages from other anatomical sites.

Materials and Methods

Mice and antibodies

C57BL/6 mice aged 8-12 weeks old (Charles River, France) were maintained under specific pathogen-free condition according to the standard housing procedure of the Erasmus MC animal care facility. Antibodies used for immunohistochemistry include the following: CD3 (17A2), F4/80 (BM8), CD11b (M1/70), purchased from eBioscience, Ly6C (ER-MP20), MARCO (ED31), purchased from Bioconnect, CD68 (FA-11), biotin-conjugated F4/80 (BM8) were purchased from Biolegend, and sialoadhesin (MOMA-1) was purchased from Acris. Antibodies used for flowcytometry were: CD3 PE (17A2), CD11b PECy7 (M1/70), CD11c APCCy7 (N418), Gr-1 APCCy7 (RB6-8C5), IL-12p40 PE (C15.6), TNF PerCPCy5.5 (MP6-XT22) were purchased from Biolegend, CD45 eFluor450 (30-F11) and CD40 PE (1C10) were from eBioscience.

Immunohistochemistry and double immunofluorescence

Liver and spleen were snap-frozen in TissueTek after removal and cryostat sections were fixed with cold acetone for 2 min. Endogenous peroxidase activity was blocked by 20 min incubation with 0.3% H_2O_2. Non-specific antibody binding was blocked with 10% rabbit serum and 5% BSA in PBS, 0.1% avidin and 0.01% biotin (DAKO) consecutively for 15 min for each blocking step. Next, tissue sections were incubated with the primary antibody (CD3, F4/80, Ly6C, CD68, CD11b, sialoadhesin and MARCO), biotin-conjugated rabbit-anti-rat Ig (DAKO) and streptavidin-HRP (DAKO) for 1 hour with proper washing after each step. The staining was visualized using diaminobenzidine (DAB, Invitrogen), and counterstained with hematoxyline (Sigma). For double immunofluorescence, goat-anti-rat Alexa594 antibody (Invitrogen) was used for visualization. Next, biotin-conjugated F4/80 was added, followed by streptavidin-FITC (Biolegend). Tissue sections were counterstained

with DAPI and were cover-slipped with Prolong-Gold (Invitrogen). Pictures were taken using Zeiss Axioplan 2 fluorescence microscope and processed with Adobe Photoshop CS3.

Isolation of total liver non-parenchymal cells, splenic and peritoneal cells

Liver was removed without perfusion, cut into small pieces, incubated in RPMI 1640 containing 30 µg/ml Liberase TM (Roche) and 20 µg/ml DNAse type I (Sigma) for 20 min, and passed through a 100 µm cell strainer. After centrifugation, cells were resuspended in PBS containing 1% FCS and 2.5 mM EDTA. Parenchymal cells were removed by low speed centrifugation at 50 g for 3 min and erythrocytes were lysed with 0.8% NH_4Cl, and remaining cells were resuspended in culture medium (RPMI 1640, 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml / 100 µg/ml Pen/Strep, 50 µM β-mercaptoethanol). Similarly, spleens were removed, treated with Liberase TM, erythrocytes were lysed, and the remaining cells were resuspended in culture medium. Peritoneal cells were obtained by washing the peritoneal cavity with cold PBS. Total liver non-parenchymal cells, splenic and peritoneal cells were used to examine cytokine production, endocytosis and ROS production.

Purification of Kupffer cells, splenic and peritoneal macrophages

Total liver non-parenchymal cells and splenic cells were blocked with antibodies against CD16/32 (2.4G2, kind gift from Dr. L. Boon, Bioceros) to decrease non-specific binding. F4/80+ cells were enriched using F4/80 APC and anti-APC beads (Miltenyi Biotech) according to the manufacturer's instructions. Next, cells were stained with CD45 eFluor450, CD3 PE, CD11b PECy7, CD11c APCCy7, GR1 APCCy7, and $CD45^+CD11c^-GR1^-CD3^-F4/80^{high}CD11b^{low}$ cells were further purified by a FACS-Aria sorter (BD), with dead cells excluded by propidium iodide. The purity of sorted Kupffer cells and splenic macrophages was routinely 95-98% and 88-90%, respectively. Peritoneal cells were incubated with antibodies against CD16/32, F4/80 APC, CD45 eFluor450, CD11b PECy7. Similarly, $CD45^+F4/80^{high}CD11b^{high}$ cells were purified and the purity of peritoneal macrophages was routinely >98%.

Culture of bone marrow-derived macrophages (BMMP)

In vitro differentiation of bone marrow cells into macrophages was performed using 10% L929-cell culture medium (conditioned-medium) as previously described (18). Briefly, bone marrow cells were isolated from femurs and tibiae, and erythrocytes were lysed. Cells were seeded in a petridish (Sarstedt) at $0.5x10^6$ cells/ml in a volume of 8 ml. After 4 days, 10 ml conditioned medium was added. On day 7, adherent cells were harvested. The purity of the F4/80+CD11b+ cells was al-

ways more than 85%. Following *in vitro* differentiation of BMMP, cells were incubated on day 7 with 50 ng/ml E. coli 055:B5 LPS (Difco) plus 50 ng/ml IFNγ (Invitrogen), or 10 ng/ml IL-4 (Biosource) as previously described (19). On day 8, adherent cells were harvested.

RNA isolation, generation of cDNA and real-time PCR

Table 1. Gene-specific primers used in RNA analysis

Gene ID	NCBI ID		Primer sequence
Fizz1	NM_020509.3	FW	tcccagtgaatactgatgaga
		RV	ccactctggatctcccaaga
Ym1	NM_009892.2	FW	catgagcaagacttgcgtgac
		RV	ggtccaaacttccatcctcca
Arg1	NM_007482.3	FW	tgacatcaacactcccctgacaac
		RV	gccttttcttccttcccagcag
IRAK-M	AF461763.1	FW	cttcccacttgaggtgaagc
		RV	atgcttggtttcgaatgtcc
tollip	NM_023764.3	FW	gcagggtgttggctatgtg
		RV	cattacagcggggctgag
tmed1	NM_010744.3	FW	gctagtcttgagaccgagtacca
		RV	gctctccaaggtgaagtcca

RNA was extracted from cells stored in RNAlater (Qiagen) using NucleoSpin RNAII kit (Bioké) and was quantified using a Nanodrop ND-1000 (Thermo). cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturers' protocol. All real time PCR reactions were performed using Bio-Rad optical 96-well plates with a MyIQ5 detection system (Bio-rad Laboratories). The probe in the master mix (TaqMan® Gene Expression Master Mix) was an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye. Primers for housekeeping gene 18S (Hs99999901_s1), TLR4 (Mm00445274_m1), TLR7 (Mm100446590_m1) and TLR9 (Mm00446193_m1) were purchased from Applied Biosystems. The nucleotide sequence of primers used to analyze the mRNA levels of GAPDH, Fizz1, Ym1, Arg1, tmed1, tollip and IRAK-M are listed in Table 1. The expression of the target genes was normalized to the expression of 18S or GAPDH using the formula $2^{-\Delta Ct}$, $\Delta Ct=Ct_{TLR}-Ct_{18S}$ or $\Delta Ct=Ct_{RNAX}-Ct_{GADPH}$.

Cytokine production by purified Kupffer cells, total liver non-parenchymal, splenic and peritoneal cells

Purified Kupffer cells were plated in a 96-well plate (Costar) at $2x10^5$/well, in 200 µl culture medium with or without LPS (*S. Minnesota* ultra pure, 100 ng/ml, Invivogen), R848 (1 µg/ml, Alexis) or CpG-1668 (5 µg/ml, Invivogen). Following overnight incubation, supernatant was harvested and

measured by ELISA for IL-10, TNF and IL-12p40 (eBioscience).

Total liver non-parenchymal, splenic cells and peritoneal cells were cultured at 1x 10^6/well in a 24-well plate (Costar) in 1 ml culture medium alone or in combination with LPS or R848. To inhibit IL-10 signaling, anti-IL-10 receptor antibodies (eBioscience) was added alone or in combination with LPS. Brefeldin A (10 µg/ml, Sigma) was added after 2 hours and cells were further incubated for another 3 hours. Following fixation and permeabilization with 2% formaldehyde and 0.5% saponin (Rectapur), intracellular cytokine staining was performed using F4/80 APC, CD45 eFluor450, CD11b PECy7, CD11c APCCy7 and IL-12p40 PE or TNF PerCPCy5.5. The frequency of TNF- or IL-12p40-positive cells was determined within the $CD45^+CD11c^-F4/80^{high}CD11b^{low}$ population for Kupffer cells and splenic macrophages and within the $CD45^+F4/80^{high}CD11b^{high}$ population for peritoneal macrophages. These marker combinations were consistently used to identify Kupffer cells, splenic and peritoneal macrophages in flow cytometric analysis.

Expression of co-stimulatory molecules

Total liver non-parenchymal, splenic and peritoneal cells were seeded in a 24-wells plate (Costar) at 1x10^6 cells/ml. The cells were incubated without or with the TLR7/8 agonist R848 (1 µg/ml, Invivogen) for 22 hours at 37°C. Expression of CD40 on Kupffer cells, splenic and peritoneal macrophages was identified by flow cytometry using F4/80 APC, CD45 eFluor450, CD11b PECy7, CD11c APCCy7 and CD40 PE. Expression level of CD40 was determined within the Kupffer cells, splenic and peritoneal macrophages.

In vitro and in vivo receptor-mediated endocytosis assay

In the *in vitro* endocytosis assay, 1x10^6 total liver non-parenchymal, splenic and peritoneal cells were incubated for 45 min with dextran-FITC (10 µg/ml, 40,000 MW, Invitrogen) or *E. coli*-FITC (2x10^8 cells/ml, Glycotope), with or without LPS or R848 at 37°C or on ice. To some conditions, the inhibitor of endocytosis, cytochalasin D (10 µM, Sigma) was added. Un-bound FITC-dextran or *E. coli*-FITC was washed away. In the *in vivo* endocytosis assay, 100 µg dextran-FITC in 200 µl PBS was injected in the tail vein. Mice were sacrificed 2 hours later and total liver non-parenchymal cells and splenic cells were isolated as previously described. In both *in vitro* and *in vivo* endocytosis assay, these cells were further stained with F4/80 APC, CD45 eFluor450, CD11b PECy7, and CD11c APCCy7. FITC positive cells were determined within the Kupffer cells, splenic and peritoneal macrophages.

ROS production assay

1x10^6 total liver non-parenchymal, splenic cells and peritoneal cells were incubated with di-

hydrorhodamine 123 (DHR123; 0.1 µg/ml, Sigma) for 15 min 37°C. Un-bound DHR123 was washed away, and cells were further incubated with PMA (50 ng/ml, Sigma), LPS, R848 or CpG-1668 for 30 min. Cells were further stained with F4/80 APC, CD45 eFluor450, CD11b PECy7 and CD11c APCCy7. ROS production was detected based on the transformation of DHR123 to rhodamine 123 (in the FL1 channel) within the Kupffer cells, splenic and peritoneal macrophages.

Statistical analysis

The average values ± SEM are presented in each graph. Statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). *P*-values were calculated by one-way analysis of variance for non-parametric data (Kruskal-Wallis test) with Dunns post test. $P<0.05$ is considered statistically significant.

Results

Detailed phenotypical analysis of liver Kupffer cells

Murine liver contains a large population of F4/80$^+$ Kupffer cells (20) with a unique irregular shape that are located almost exclusively in the sinusoidal compartment and distributed evenly across the tissue section (Figure 1A). Additional immunohistochemical analysis showed that in the liver, the frequency of cells expressing other macrophage-markers, such as CD11b, CD68 and sialoadhesin, is relatively high, whereas the frequency of MARCO-expressing cells is low (Supplementary Figure S1). As shown in Figure 1B, further analysis with immunofluorescence showed that all F4/80$^+$ cells co-express sialoadhesin. CD68+ cells are more frequent than F4/80-expressing cells, distributed across the liver section and appear as both large cells with irregular shape and small, round cells (Figure 1B, upper panel). Approximately 80% of the F4/80+ cells co-express CD68, hence F4/80 and CD68 expression only partially overlap by histology. In contrast, CD11b+ cells are less frequent than F4/80+ cells; and approx. 30% of all cells co-express F4/80 and CD11b by immunofluorescence on tissue sections (Figure 1B, lower panel). Further evaluation of the phenotype of murine Kupffer cells by flow cytometry showed one population of cells that was defined as CD45$^+$CD11c$^-$F4/80^{high}CD11b^{low}, which appears large and contains multiple phagocytic vacuoles with macrophage morphology (Figure 1C). Another population contains the CD45$^+$CD11c$^-$F4/80^{low}CD11b^{high} cells, which are smaller, contain fine granules in the cytoplasm, and resemble monocytes. The double-negative population resembles lymphocytes: round, small cells with large nuclei and a relatively small volume of cytoplasm. Compared to isolated splenic macrophages, which primarily represent red pulp macrophages, the levels of expression of CD11b are higher on Kupffer cells and peritoneal macrophages (Supplementary Figure S2). Based on this observation, we find that the frequency of Kupffer cells, defined as CD45$^+$CD11c$^-$F4/80^{high}CD11b^{low} cells in the liver is 7% of the CD45+ leukocytes.

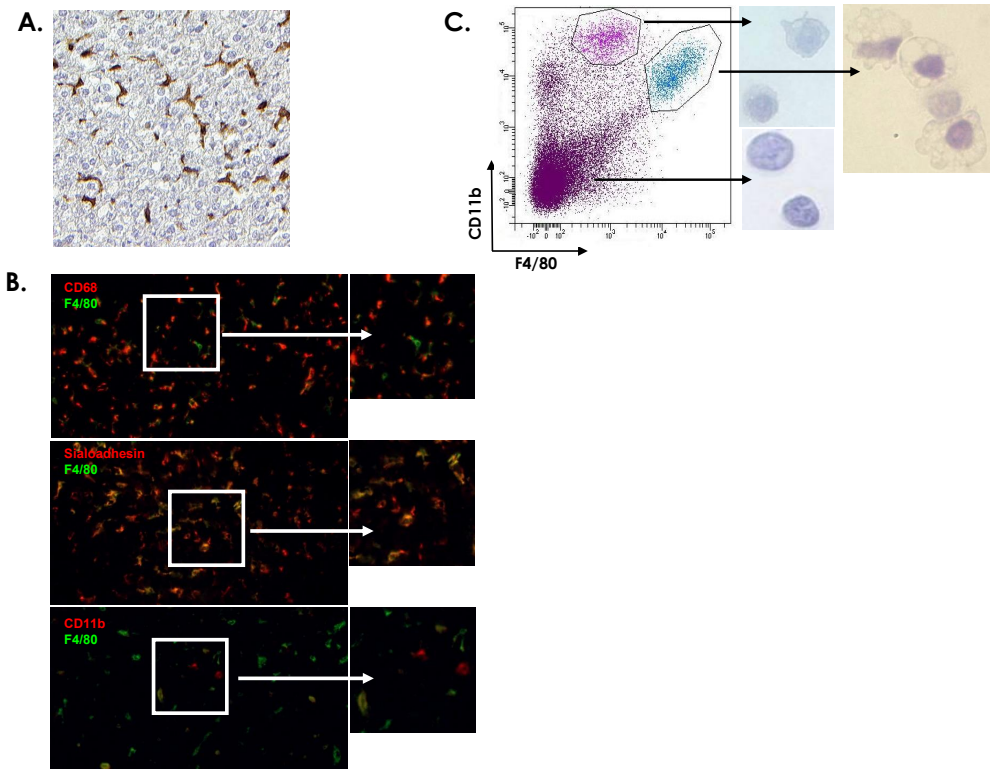

Figure 1. Identification of Kupffer cells by immunohistochemistry, double immunofluorescence and flow cytometry. A) Murine Kupffer cells are identified by F4/80 staining and appear as large cells with projections between the hepatocytes. B) *Upper panel*: The liver contains numerous CD68+ cells, which partly co-express F4/80. *Middle panel*: The majority of F4/80+ cells co-express sialoadhesin. *Lower panel*: Part of F4/80+ cells also express CD11b. C) Kupffer cells are identified as F4/80^{high}CD11b^{low} cells and contain vacuoles. Liver tissue sections and cell suspensions were prepared from C57BL/6 mice. The stainings for immunohistochemistry and flow cytometry were performed five times; and double immunofluorescence was performed two times, with similar results.

Kupffer cells and splenic macrophages have comparable mRNA levels of TLR4, TLR7/8 and TLR9

Murine macrophages have the ability to sense LPS, single-stranded RNA and DNA containing CpG motifs via TLR4, TLR7/8 and TLR9, respectively (10). To determine the basal levels of TLR4, TLR7/8 and TLR9 mRNA on Kupffer cells, we performed a two-step approach in which we first enriched the liver suspension for F4/80+ cells (Figure 2A, upper panel), and then sorted for F4/80^{high}CD11b^{low} cells by flow cytometry resulting in 95-98% pure Kupffer cells (Figure 2A, lower panel). Splenic macrophages were isolated similarly by F4/80+ cells enrichment (Figure 2A, upper panel), resulting in 90% purity after FACS sorting (Figure 2A, lower panel). Total peritoneal cells

consisted, on average, of 35% peritoneal macrophages (Figure 2A, upper panel) and FACS sorting resulted in 99% purity (Figure 2A, lower panel). As shown in Figure 2B, mRNA analysis of BMMP, purified resting Kupffer cells, splenic and peritoneal macrophages shows that TLR4, TLR7/8 and TLR9 mRNA are expressed by all cell types and the levels of expression are slightly differ.

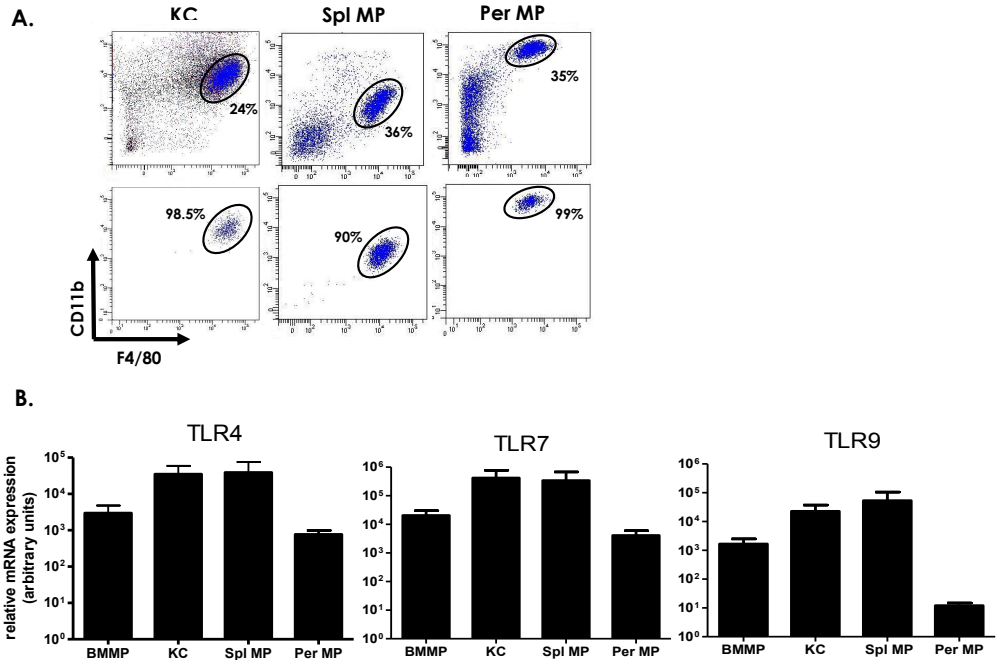

Figure 2. Differential expression levels of TLR4, TLR7/8 and TLR9 mRNA by Kupffer cells, splenic and peritoneal macrophages. A) Representative dotplots to depict the purification of Kupffer cells, splenic and peritoneal macrophages as described in Materials and Methods, and used for the detection of basal TLR4, TLR7/8 and TLR9 mRNA**.** *Upper panel:* dotplots are shown following F4/80+ cell enrichment of Kupffer cells and splenic macrophages, and without enrichment of peritoneal cells. *Lower panel:* dotplots of $CD45^{+}C-D11c^{-}GR1^{-}CD3^{-}F4/80^{high}CD11b^{low}$ Kupffer cells and splenic macrophages, and $CD45^{+}F4/80^{high}CD11b^{high}$ peritoneal macrophages, following FACS sorting. The experiments were performed 4 times with similar results. All percentages depicted are positive cells within the CD45+ gate. B) The levels of TLR4, TLR7/8 and TLR9 mRNA from sorted Kupffer cells, splenic and peritoneal macrophages are compared to culture-derived BM-MP. 18s ribosomal RNA was used for normalization. The assays were performed 4 times.

Kupffer cells produce IL-10 following TLR4, TLR7/8 and TLR9 ligation

To determine whether the expression levels of TLR mRNA on murine Kupffer cells are reflected functionally by their responsiveness to TLR ligation, we determined cytokine production by highly purified Kupffer cells upon stimulation with TLR agonists. As shown in Figure 3A, purified Kupffer cells spontaneously release IL-10 (average of 105 pg/ml) and, upon TLR4, TLR7/8 and TLR9 ligation, show a mild increase of the levels of IL-10 detected in supernatant (average: 180, 210 and 130 pg/ml, respectively). Besides IL-10, we also measured IL-12p40 and TNF upon TLR ligation on Kupffer cells.

Under all conditions tested, the levels of IL-12p40 and TNF in supernatant are low or undetectable. The low or undetectable levels of IL-12p40 and TNF are not only observed upon stimulation of purified Kupffer cells, but also when the frequency of cytokine-producing cells is determined by flow cytometry using total organ cell suspensions. Upon stimulation with TLR4, TLR7/8 and TLR9 agonists, and subsequent gating on Kupffer cells and splenic macrophages in our flow cytometric analysis, we observed low to undetectable frequencies of TNF- and IL-12p40-producing cells (Figure 3B). The frequency of TNF- and IL-12p40-producing Kupffer cells and splenic macrophages was not increased by blocking the inhibitory function of IL-10 using anti-IL10R antibodies (data not shown). In comparison, following TLR4 and TLR7/8 ligation of peritoneal macrophages, relatively high frequencies of TNF-producing cells (46% and 30%, respectively) and low frequencies of IL-12p40-producing cells (both 1.8%) are observed. These findings demonstrate that expression levels of TLR4, TLR7/8 and TLR9 mRNA do not necessary reflected in their responsiveness to TLR ligation in term of cytokine production by Kupffer cells.

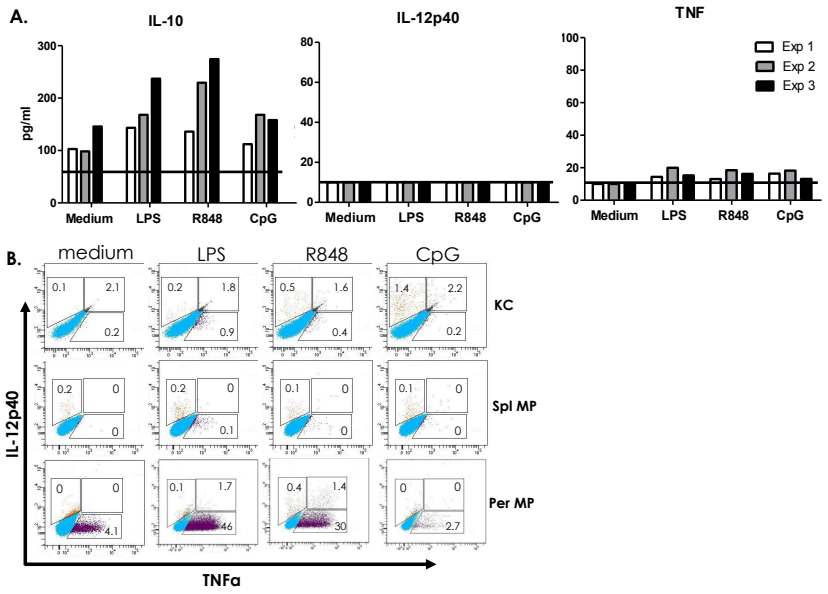

Figure 3. Liver Kupffer cells produce IL-10 upon TLR4, TLR7/8 and TLR9 ligation. A) Levels of IL-10, IL-12p40 and TNF produced by Kupffer cells. 5x10^4 FACS-sorted Kupffer cells (purity: 99%) were stimulated overnight with LPS, R848 or CpG-1668 in a volume of 200 µl. IL-10, TNF and IL-12p40 levels in supernatant were measured by ELISA. The sensitivity of the ELISA was 70, 10 and 10 pg/ml for IL-10, IL-12p40 and TNF, respectively. Following LPS, R848 and CpG-1668 stimulation, Kupffer cells weakly increase their IL-10 production whereas IL-12p40 and TNF production are low or undetectable. The experiment was repeated 3 times. B) A representative figure of intracellular cytokine production using total liver non-parenchymal cells, splenic and peritoneal cells. Cells were incubated with medium, LPS, R848 and CpG-1668 in the presence of brefeldin A for 5 hours, fixed and subjected to intracellular cytokine staining. Kupffer cells, splenic and peritoneal macrophages were gated and analyzed for TNF and IL-12p40 production. Control staining without IL-12p40 and TNF antibodies was used to set the threshold gate. The frequency of IL-12p40- and TNF-producing Kupffer cells and splenic macrophages are relatively low or not detected under all conditions tested. High frequencies of TNF-producing peritoneal macrophages are observed following TLR4 and TLR7/8, but not TLR9 ligation. The assay was performed 4 times with similar results.

Kupffer cells and splenic macrophages up-regulate the expression of co-stimulatory molecules upon TLR7/8 ligation

Since Kupffer cells are known to express co-stimulatory molecules on their cell surface (21), we examined whether TLR4, TLR7/8 and TLR9-stimulated Kupffer cells upregulate their expression of co-stimulatory molecules. As shown in Figure 4, upon TLR7/8 ligation, a two-fold and four-fold increase of CD40 expression is detected on Kupffer cells and splenic macrophages, respectively, whereas peritoneal macrophages exhibited no enhanced expression. Moreover, we observed a high basal expression of MHC II on Kupffer cells, which was not further up-regulated by TLR ligation (data not shown). No up-regulation of CD80 and CD86 following TLR4, TLR7/8 and TLR9 ligation, or CD40 following TLR4 and TLR9 ligation was observed (data not shown).

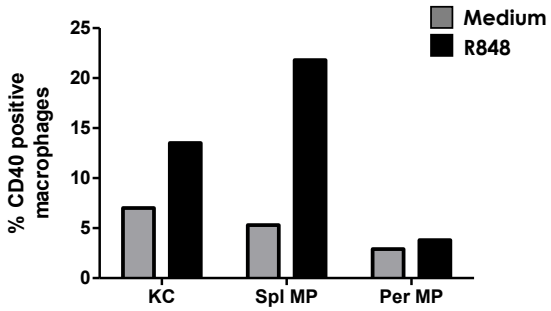

Figure 4. Kupffer cells respond to TLR7/8 ligation and up-regulate CD40 expression. Total liver non-parenchymal cells, splenic and peritoneal cells were incubated with medium or R848 overnight. By flow cytometry, the frequencies of CD40-expressing cells were analyzed on Kupffer cells, splenic and peritoneal macrophages. Control staining without the addition of CD40 antibody was used to set the threshold gate. Results from a representative experiment are depicted. Following TLR7/8 ligation, Kupffer cells and splenic macrophages, but not peritoneal macrophages, up-regulate CD40 expression (observed in 4 independent experiments). The assay was performed 4 times for Kupffer cells and splenic macrophages, and 3 times for peritoneal macrophages.

Kupffer cells have higher mRNA levels of tmed1, tollip and IRAK-M than peritoneal macrophages

Since low or undetectable levels of TNF and IL-12p40 were observed in Kupffer cells upon TLR ligation, we wondered whether the intrinsic characteristics of resting Kupffer cells resembled those of LPS/IFNγ-activated or IL-4-activated macrophages, the so-called M1 and M2 macrophages, respectively (19), by determining the expression levels of Ym1, Fizz1 and Arg1 (22, 23). As shown in Figure 5A, mRNA analysis on sorted Kupffer cells, splenic and peritoneal macrophages showed that Kupffer cells, as well as splenic macrophages, have low mRNA levels of Ym1, Fizz1 and Arg1. Peritoneal macrophages exhibit a distinct expression profile with low mRNA levels of Ym1 and Arg1, but high mRNA expression of Fizz1.

The role of negative regulators of TLR signalling in Kupffer cells and Peyer's patch DC have been described (24, 25). The mRNA levels of tmed1, tollip and IRAK-M in sorted Kupffer cells, splenic and peritoneal macrophages were analysed. We observed that Kupffer cells, as well as splenic macrophages, have higher mRNA levels of tmed1, tollip and IRAK-M than peritoneal macrophages (Figure 5B). The high expression levels of these negative regulators in Kupffer cells and splenic macrophages, in comparison to peritoneal macrophages, may contribute to the weak responsiveness of Kupffer cells and splenic macrophages to TLR agonists.

Kupffer cells have higher dextran-endocytic activity than splenic and peritoneal macrophages

The hallmark feature of macrophages is to eliminate particulates or microbes by endocyto-

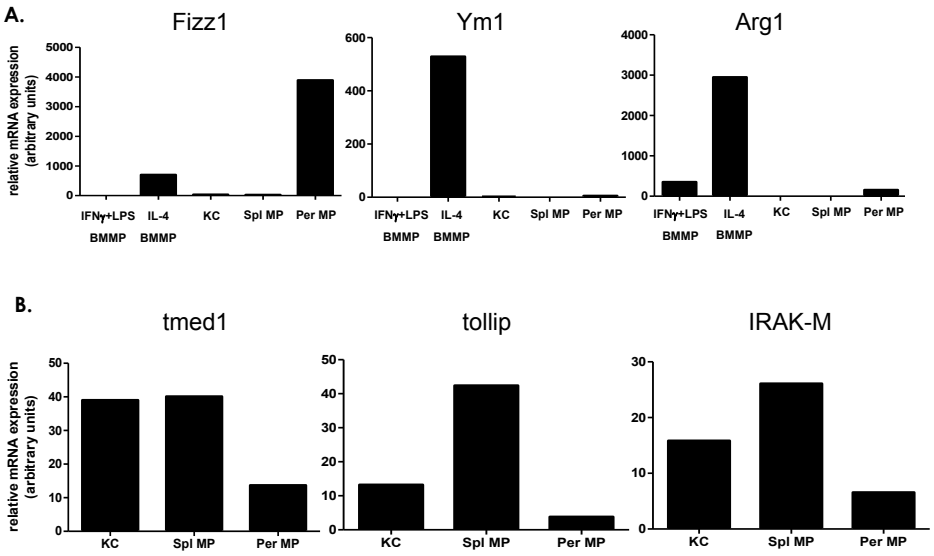

Figure 5. Kupffer cells have high mRNA levels of tmed1, tollip and IRAK-M. Kupffer cells, splenic and peritoneal macrophages were purified as described in Materials and Methods and were used to detect the basal mRNA levels of Fizz1, Ym1, Arg1, tmed1, tollip and IRAK-M. GAPDH was used for normalization. A) mRNA levels of Fizz1, Ym1 and Arg1 of purified Kupffer cells, splenic and peritoneal macrophages. IFNγ and LPS-stimulated BMMP and IL-4-stimulated BMMP were prepared as described in Materials and Methods and used as controls to detect the mRNA levels of Fizz1, Ym1 and Arg1. On the basis of the mRNA pattern of Fizz1, Ym1 and Arg1, Kupffer cells and splenic macrophages more resemble IFNγ and LPS-stimulated BMMP than IL-4-stimulated BMMP. B) mRNA levels of tmed1, tollip and IRAK-M of Kupffer cells, splenic and peritoneal macrophages. Kupffer cells, as well as splenic macrophages, possess higher mRNA levels of tmed1, tollip and IRAK-M than peritoneal macrophages. Splenic macrophages exhibit higher mRNA levels of tollip and IRAK-M than Kupffer cells and peritoneal macrophages. Both assays were performed 2 times with similar results.

sis (26). We therefore examined the ability of Kupffer cells to endocytose dextran and *E. coli* bacteria as compared to splenic macrophages and peritoneal macrophages. As shown in Figure 6A and 6B, we observed that liver Kupffer cells have a higher ability to endocytose dextran than splenic macrophages (Figure 6B: 47% and 15%, respectively). Furthermore, their ability to endocytose is more potent than of peritoneal macrophages (Figure 6B: 30%). A similar result was observed when endocytosis of dextran was examined *in vivo*. As shown in Figure 6C, Kupffer cells exhibit a more potent ability to take up dextran than splenic macrophages (95% and 13%, respectively). Interestingly, we observe that peritoneal macrophages are very efficient to endocytose *E. coli* in comparison with Kupffer cells and splenic macrophages (Figure 6A and 6B). Similar as dextran-uptake, the ability to endocytose *E. coli* is higher for Kupffer cells than splenic macrophages (Figure 6B: 59% versus 22%, respectively). Incorporation of cytochalasin D, a specific inhibitor for endocytosis, reduces both dextran and *E. coli* up-take (data not shown). Furthermore, we examined whether TLR ligation modulates endocytosis by Kupffer cells, and whether the effect reflected the expression of TLR

mRNA. As for TLR-induced cytokine production, we observed that TLR4, TLR7/8 and TLR9 ligation did not affect the degree of endocytosis by Kupffer cells (Supplementary Figure 3A).

Kupffer cells exhibit relatively higher spontaneous ROS production compared to splenic and peritoneal macrophages

Following endocytosis, elimination of particulates and pathogens by macrophages requires the generation of oxygen species (27). As shown in Figure 7, we observe 28% ROS-positive Kupffer cells without *in vitro* stimulation, indicating that Kupffer cells spontaneously produce ROS. Basal ROS production by Kupffer cells is higher than by splenic and peritoneal macrophages (8% and 7% ROS-positive, respectively; Figure 7B). PMA stimulation of Kupffer cells only mildly increases ROS production as compared to the PMA-induced increase observed for splenic and peritoneal macrophages. Importantly, TLR4, TLR7/8 and TLR9 ligation, alone or in combination with *E. coli*, do not further enhance ROS production by PMA-stimulated Kupffer cells (Supplementary Figure 3B).

Discussion

In this study, we demonstrate that, under steady state conditions, mouse Kupffer cells are endocytic, whereas TLR-induced cytokine production is minimal despite the TLR mRNA expression. This combination of features makes Kupffer cells functionally distinct from splenic and peritoneal macrophages. Our observations show that Kupffer cells share more functional similarities to splenic macrophages, in line with the notion that both represent tissue-resident macrophages of blood-filtering organs, while they are more distinct to resident peritoneal macrophages.

Here we show that even though Kupffer cells are weak producers of cytokines upon TLR ligation, they exhibit potent dextran-endocytic activity compared to splenic and peritoneal macrophages. It has been shown before that Kupffer cells, splenic as well as peritoneal macrophages are endocytic (14, 28-30). In this respect, it was demonstrated that Kupffer cells act as non-specific phagocytes with high peroxidase activity and ability to remove latex beads, colloidal carbon, and erythrocytes (31-34). It is remarkable that the dextran-endocytic capacity of Kupffer cells exceeds that of splenic macrophages. In line with previous studies (35, 36), this observation points towards a more functional specialization of Kupffer cells to take up and eliminate foreign bodies and debris from blood circulation in comparison to splenic macrophages. In keeping with their function, we demonstrate that a significant proportion of Kupffer cells express sialoadhesin and MARCO, among other scavenger receptors. In comparison, these receptors are expressed on only minor subsets of splenic macrophages, namely marginal zone metallophillic and marginal zone macrophages, respectively (29), and are shown to be involved in elimination of *E. coli, Staphylococcus aureus* and *Neisseria meningitides* (37-39). The

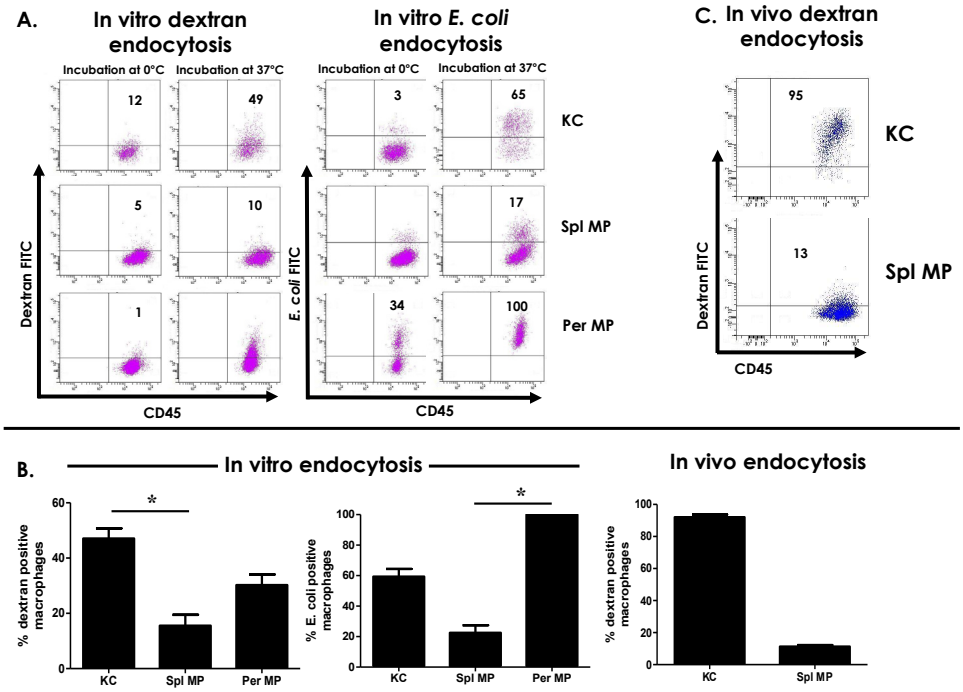

Figure 6. Kupffer cells have potent dextran-endocytic activity. A) A representative figure of endocytic activity of Kupffer cells, splenic and peritoneal macrophages. Total liver non-parenchymal cells, splenic and peritoneal cells were incubated with either dextran-FITC or *E. coli*-FITC for 45 min and their uptake by Kupffer cells, splenic and peritoneal macrophages were analyzed by flow cytometry. Control staining without the addition of dextran-FITC or *E. coli*-FITC was used to set the threshold gate. Liver Kupffer cells show the strongest activity in receptor-mediated dextran-endocytosis, compared to splenic or peritoneal macrophages. Peritoneal macrophages have a higher *E. coli*-endocytic activity than Kupffer cells and splenic macrophages. The assay was performed 4 times with similar results. B) Graphic representation of dextran-FITC or *E.coli*-FITC positive macrophages. Kupffer cells and peritoneal macrophages have a higher dextran-endocytic and E. coli-endocytic activity than splenic macrophages. C) Representative results of *in vivo* dextran-endocytic activity by Kupffer cells and splenic macrophages are depicted. Mice were injected with 100 µg dextran-FITC in 200 µl PBS via the tail vein. Mice were sacrificed 2 hours later and total liver non-parenchymal cells and splenic cells were isolated as described in Materials and Methods. Kupffer cells show a stronger dextran-endocytic activity compared to splenic macrophages. The assay was performed 3 times with similar results.

presence of sialoadhesin and MARCO on Kupffer cells may partially contribute to their high endocytic function even though further studies need to be done to show their relative contribution.

Besides potent endocytic activity, we further demonstrate that Kupffer cells and splenic macrophages show comparable expression levels of TLR4, TLR7/8 and TLR9 mRNA, and that triggering of the receptors by TLR ligation does not result in substantial up-regulation of neither co-stimulatory molecules nor cytokine production. The low or undetectable levels of pro-inflammatory cytokines upon TLR triggering on Kupffer cells and red pulp splenic macrophages may relate to their function to maintain ho-

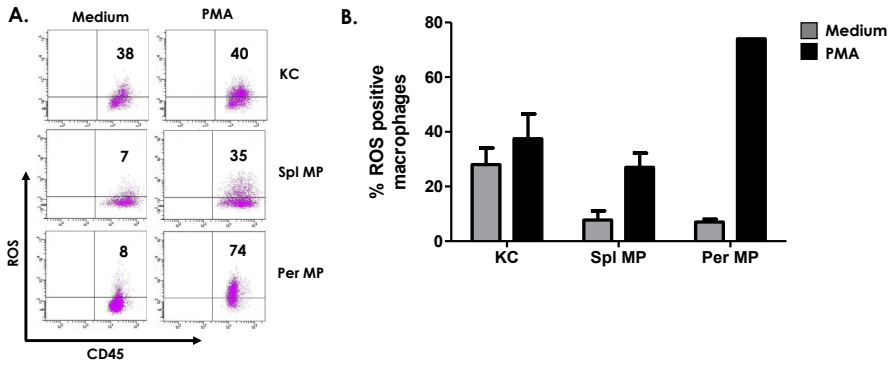

Figure 7. Kupffer cells exhibit relatively high spontaneous ROS production compared to splenic and peritoneal macrophages. Total liver non-parenchymal cells, splenic and peritoneal cells were incubated for 30 min with medium or PMA, and ROS production was analyzed within the Kupffer cells, splenic and peritoneal macrophages by flow cytometry. Control staining without the addition of DHR123 was used to set the threshold gate. A) A representative figure of ROS-positive Kupffer cells, splenic and peritoneal macrophages. A higher frequency of ROS-positive Kupffer cells is observed under basal conditions compared to splenic or peritoneal macrophages. The assay was performed 4 times for Kupffer cells and splenic macrophages, and 2 times for peritoneal macrophages, with similar results. B) Graphic representation of ROS-positive macrophages from the liver, spleen and peritoneum. Splenic and peritoneal macrophages are more responsive to PMA stimulation to produce ROS than Kupffer cells.

meostasis following contact with foreign substances from the circulation. On the basis of the expression of Ym1, Arg1 and Fizz1 mRNA, Kupffer cells do not resemble the IL-4-stimulated macrophages. Peritoneal macrophages potently produce TNF following TLR4 and TLR7/9 ligation. This can be explained by the limited exposure of these cells to particularly gut-derived antigens, resulting in a higher sensitivity upon ligation of pattern recognition receptors. It is not possible to detect the TLR4, TLR7/8 and TLR9 at the protein level due to the absence of good commercially available antibodies for this purpose. The discrepancy between the high levels of TLR mRNA and the selective induction of co-stimulatory molecules and low inducible cytokine production may be related to the induction of immunosuppressive cytokines (6, 18)), although we observed that IL-10 does not suppress TNF production by Kupffer cells and splenic macrophages. The weak responsiveness to TLR ligation may also due to the presence of negative regulators downstream of the TLRs.

In this respect, it has been shown that negative regulators, such as IRAK-M, sigirr, tollip and tmed1 are up-regulated in Kupffer cells and Peyer's patch DC due to the constant exposure to low doses of LPS, rendering these cells less responsive to LPS (24, 25). Indeed Kupffer cells, as well as splenic macrophages, possess high mRNA levels of tmed1, tollip and IRAK-M in comparison to peritoneal macrophages. The high mRNA levels of these negative regulators may provide a mechanistic explanation by which Kupffer cells and splenic macrophages regulate their response towards endocytosed-microorganisms or antigens.

As opposed to a recent study, we identified only one macrophage population in the liver (14). This previous study identified two subpopulations of Kupffer cells based on the expression of F4/80, CD11b and CD68. Only one population, F4/80+CD68+ Kupffer cells, was shown to be endocytic and able to up-regulate ROS production following ATP stimulation (14). The fact that liver perfusion was performed in this study suggests that the second population, the F4/80+CD11b+, represents marginating cells, whereas in our study, the F4/80^{low}CD11b^{high} cells resemble monocytes and their frequency is strikingly increased during inflammation (data not shown) (40, 41). Furthermore, it has been shown that these cells are less sensitive to gadolinium chloride- or clodronate liposome-mediated depletion, and have a lower ability to produce reactive oxygen and nitrogen species. This suggests a low endocytic and enzymatic activity by these cells (data not shown, (42, 43)) which contradicts the hallmark function of Kupffer cells. Based on our results, in combination with previous studies, we suggest that the F4/80^{low}CD11b^{high} cells are not tissue-resident, but newly recruited or inflammatory monocytes.

In this regard, it is important to mention that previously, small and large murine Kupffer cells have been described, with the large Kupffer cells being more endocytic and producing more oxygen species than the small ones (42, 43). Partly in line with Kinoshita et al, Kono et al and He et al, we observe that the F4/80^{high}CD11b^{low} Kupffer cells, which are larger in size, have higher endocytic activity and basal ROS content than the F4/80^{low}CD11b^{high} monocytic population (Fig. 5, Fig. 6, data not shown). Together, our findings do not substantiate the identification of multiple, distinct populations of Kupffer cells in healthy mouse liver. Phenotypically, however, heterogeneity exists among these cells with respect to the expression levels of CD11b, CD68, sialoadhesin and MARCO, as evident from histological evaluation. Whether this heterogeneity reflects a spectrum induced by variations in micro-environmental or maturational differences or genuine subsets of Kupffer cells remains to be determined.

Taken together, our study shows that Kupffer cells have a unique phenotypical and functional combination, which strongly relates to the function of the liver. We observe more similarity between Kupffer cells and splenic macrophages, which are both tissue-fixed and blood-filtering macrophages, in contrast to the free-moving resident peritoneal macrophages. Significant endocytic activity by Kupffer cells in the absence of TLR-induced cytokine production may be beneficial to enhance elimination of pathogens, apoptotic cells and debris without inducing liver injury by pro-inflammatory mediators.

Acknowledgement

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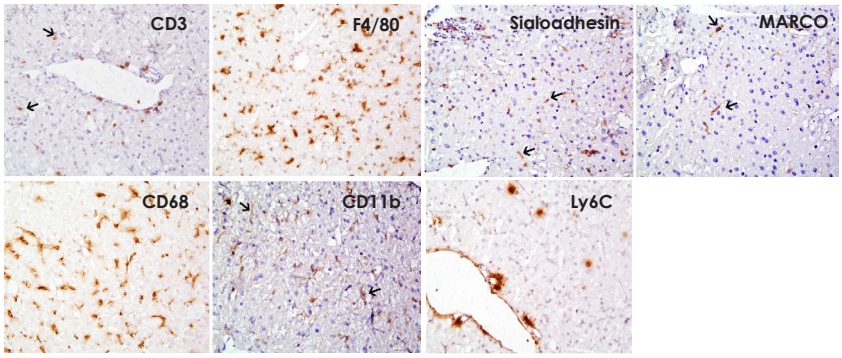
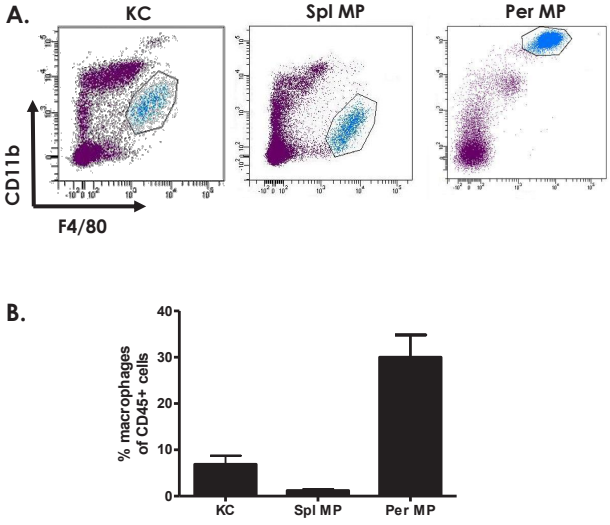
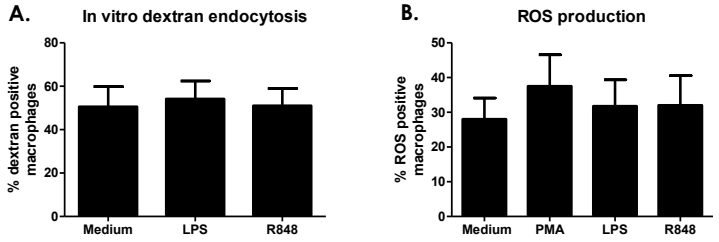
Supplementary Data



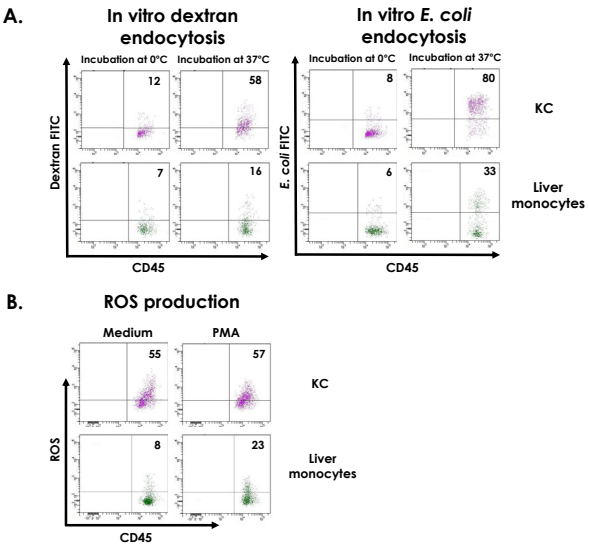
Supplementary Figure S1. The expression of various scavenger receptors and adhesion molecules in the liver. Representative liver tissue sections obtained from C57BL/6 mice and stained for F4/80, sialoadhesin, MARCO, CD68, CD11b, Ly6C, and CD3 as a control. As expected, only few CD3+ cells are observed in the healthy mouse liver. F4/80+, CD11b+, CD68+ and sialoadhesin+ cells are present in the liver, while only few MARCO+ cells are observed. Based on the single IHC staining, double immunofluorescence stainings of F4/80-CD11b, F4/80-CD68 and F4/80-sialoadhesin were performed (shown in Figure 1B). Both IHC and immunofluorescence were performed at least 2 times with similar results.



Supplementary Figure S2. Flow cytometric identification of macrophages from the liver, spleen and peritoneum by F4/80 and CD11b expression. Total liver non-parenchymal cells, splenic and peritoneal cells were analyzed for F4/80 and CD11b expression. For the liver and spleen, CD11c+ cells were excluded. A) A representative FACS plot showing differential expression of CD11b by Kupffer cells, splenic and peritoneal macrophages. Peritoneal macrophages have the highest CD11b expression, compared to Kupffer cells and splenic macrophages. B) Graphic representation of F4/80^{high}CD11b^{low} Kupffer cells and splenic macrophages, and F4/80^{high}CD11b^{high} peritoneal macrophages by flow cytometry. The experiments were performed 4 times with similar results.



Supplementary Figure S3. TLR4 and TLR7/8 ligation do not induce endocytosis and ROS production in Kupffer cells. A) A graphic representation of dextran-endocytosis by Kupffer cells upon TLR4 and TLR7/8 ligation. Total liver non-parenchymal cells were incubated with dextran-FITC for 45 min and their uptake by Kupffer cells was analyzed by flow cytometry. Control staining without the addition of dextran-FITC was used to set the threshold gate. LPS and R848 stimulation do not increase the endocytic ability of Kupffer cells. The assay was performed 3 times with similar results. B) A graphic representation of ROS production by Kupffer cells upon TLR4 and TLR7/8 ligation. Total liver non-parenchymal cells were incubated for 30 min with medium, PMA, LPS or R848, and ROS production by Kupffer cells was analyzed by flow cytometry. Control staining without the addition of DHR123 was used to set the threshold gate. LPS and R848 do not enhance ROS production in Kupffer cells. The assay was performed 2 times with similar results.



Supplementary Figure S4. Liver monocytic cells have a lower endocytic activity and ROS production than Kupffer cells. A) A flow cytometric representation of dextran- and *E. coli*-endocytosis by Kupffer cells and liver monocytic cells. Total liver non-parenchymal cells were incubated with dextran-FITC and *E.coli* FITC for 45 min and their uptake by Kupffer cells and liver monocytic cells was analyzed by flow cytometry. Control staining without the addition of dextran-FITC or *E.coli* FITC was used to set the threshold gate. Liver monocytic cells have lower dextran- and *E. coli*-endocytic activities than Kupffer cells. The assay was performed 4 times with similar results. B) A flow cytometric representation of ROS production by Kupffer cells and liver monocytic cells upon PMA stimulation. Total liver non-parenchymal cells were incubated for 30 min with medium or PMA, and ROS production by Kupffer cells and liver monocytic cells was analyzed using flow cytometry. Control staining without the addition of DHR123 was used to set the threshold gate. Liver monocytic cells have a lower basal level of ROS than Kupffer cells, and are more responsive to PMA stimulation to increase their ROS production. The assay was performed 3 times with similar results.

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4

The DNA-binding factor CTCF critically controls gene expression in macrophages

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Abstract

Macrophages play an important role in immunity and homeostasis. Upon pathogen recognition via specific receptors, they rapidly induce inflammatory responses. This process is tightly controlled at the transcriptional level. The DNA binding zinc-finger protein CCCTC-binding factor (Ctcf) is a crucial regulator of long-range chromatin interactions and coordinates specific communication between transcription factors and gene expression processes. In this study, the *Ctcf* gene was specifically deleted in myeloid cells by making use of the transgenic Cre-LoxP system. Conditional deletion of the *Ctcf* gene in myeloid cells induced a mild phenotype *in vivo*. *Ctcf*-deficient mice exhibited significantly reduced expression of MHC class II in the liver. *Ctcf*-deficient macrophages demonstrated a normal surface phenotype and phagocytosis capacity. Upon TLR stimulation they produced normal levels of the pro-inflammatory cytokines IL-12 and IL-6, but manifested a strongly impaired capacity to produce TNF and IL-10, as well as to express the IL-10 family members IL-19, IL-20 and IL-24. Taken together, our data demonstrate a role of Ctcf that involves fine-tuning of macrophage function.

Introduction

Cells belonging to the myeloid lineage take a central role in homeostasis and immunity, and are involved in the initiation, maintenance and resolution of immune responses. Myeloid cells include granulocytes, monocytes, dendritic cells and macrophages (1). These cells recognize bacteria, viruses or apoptotic cells via a broad array of pattern recognition receptors to trigger their effector functions leading to elimination of bacteria and viruses or removal of apoptotic cells (1). Recognition via specific receptors, such as Toll-like receptors (TLR), and phagocytic uptake of pathogens by macrophages or dendritic cells (DC) generally induces the production and secretion of pro-inflammatory cytokines, such as TNF, IL-6 or IL-12, that initiate and promote host defense. Cytokine expression is generally inducible and can be cell-type specific (2). Inflammatory responses required for pathogen elimination are extremely complex and tightly controlled at the level of gene transcription. Transcriptional programs need to initiate an efficient effector response that controls the harmful challenge to the host.

The DNA-binding CCCTC binding factor (Ctcf) plays an important role in the regulation of expression of numerous genes, and approximately 14,000 - 40,000 binding sites have been identified genome-wide (3, 4). Ctcf is a highly conserved 11-zinc finger protein involved in the regulation of gene expression in a cell-type specific manner at complex gene clusters, such as the β-globin, MHC class, and the Ig gene loci (3, 4). Moreover, Ctcf is important in a variety of regulatory functions, including genomic imprinting, X-chromosome inactivation and long-range chromatin interactions, hormone-responsive gene silencing, enhancer blocking and/or barrier gene insulation and transcriptional activation or repression (3, 4). The distinct functions of Ctcf are exerted by combinatorial use of 11-zinc fingers allowing it to bind highly divergent sequences (3). Ctcf regulates chromatin architecture together with cohesin proteins, which are enriched at Ctcf -binding sites (5-7). In addition, multiple functions of Ctcf are enabled by interaction with different binding partners like transcription factors (Yy1, Kaiso and YB-1) (8-10), chromatin modifying proteins (Sin3a, CHD8, Suz12) (11-13), RNA polymerase II (14) and poly(ADP-ribose) polymerase-1 (Parp-1) (15).

Recently, Ctcf was found to control MHC class II gene expression (16), and it was reported that enforced overexpression of Ctcf in DC caused increased apoptosis, reduced proliferation and impaired differentiation (17). Conditional targeting experiments in mice showed that Ctcf controls both T cell development (18) and differentiation into effector subsets, particularly into type T helper (Th)-2 cells (19). We previously reported that mice with a Ctcf defect in $CD4^+$ T cells exhibit reduced Th2 development and production of the Th2 cytokines IL-4, IL-5 and IL-13, despite the expression of normal levels of the key Th2 transcription factors Gata3 and Satb1 (19). Interestingly, it was de-

monstrated that cooperation between T-bet and Ctcf is required for Th1 cell-specific expression of IFNγ (20). In addition, Ctcf also plays a role in the regulation of V(D)J recombination events and V gene usage at the Ig H and L chain loci in B cells (21, 22). The role of Ctcf in development and function of myeloid cells in vivo has not been investigated. Here, we used the transgenic Cre-loxP system to generate conditional myeloid-specific Ctcf-knockout mice, allowing analysis of the role of Ctcf in the development and function of macrophages in vivo and in vitro. We demonstrate that deletion of the Ctcf gene in cells with active Cre expression driven by the M lysozyme (LysM) promoter affected the numbers of macrophages generated in from monocytes. Interestingly, we observed that Ctcf-deficient macrophages from *LysM-Cre Ctcf$^{f/f}$* mice retained the capacity to produce IL-6 and IL-12 upon TLR ligation, but manifested impaired expression or production of the regulatory cytokines of the IL-10 family as well as TNF.

Materials and Methods

To obtain mice with conditional knock-out of CTCF in macrophages, mice bearing the CTCF allele flanked with loxP sites (Ctcf^{f} mice) (18) were crossed with mice expressing Cre recombinase under the LysM promoter (LysM-Cre mice) (23). To investigate the efficiency of Cre-mediated deletion in the various myeloid cell lineages, we used a Cre-reporter strain harboring a targeted insertion of enhanced yellow fluoresencent protein (EYFP) into the ROSA26 locus (24). Genotyping of mice for the presence of transgenic constructs was performed as previously described (18, 23), (24). Crosses of LysM-Cre transgenic and Ctcf$^{f/f}$ mice yielded mice with myeloid-specific deletion of the *Ctcf* gene (*LysM-Cre Ctcf$^{f/f}$*) mice, as well as littermates that did not have the LysM-Cre transgene or a floxed *Ctcf* gene, both of which were referred to as "wild type" mice. Mice were bred and maintained at the Erasmus MC animal facility under specific pathogen-free conditions, and used for experiments at 6–12 weeks of age. Experimental procedures were approved by the Erasmus University committee of animal experiments.

Preparation of single cell suspensions

Single-cell suspensions were prepared using standard methods and filtered through a 100 µm cell strainer. Livers were removed without perfusion; small pieces were incubated for 30 min in RPMI-1640 containing 30 µg/ml Liberase TM (Roche) and 20 µg/ml DNAse type I (Sigma), and passed through a 100 µm cell strainer. Cells were resuspended in PBS containing 1% serum and 2.5 mM EDTA. Parenchymal cells were removed by low speed centrifugation at 300 rpm for 3 min, and erythrocytes were lysed with 0.8% NH_4Cl. Remaining liver cells were resuspended in culture medium.

Phenotypic analysis by flow-cytometry

Aliquots of $2x10^6$ cells were incubated with a cocktail of monoclonal antibodies (mAb). Each incubation step was performed at 4°C for 30 min and cells were subsequently washed two times in FACS buffer: PBS supplemented 1% FCS, 2.5 mM EDTA and 0.1% sodium azide. Prior to acquisition, labeled cells were incubated for 1 min with propidium-iodide (Sigma), 7-AAD (Invitrogen) or DAPI (Molecular Probes) at the final concentration of 1 µg/ml and washed with FACS buffer. Ly6G-PE, Ly6C-FITC/-biotin antibodies were purchased from BD Pharmingen. Antibodies against CD4-FITC, CD86-FITC, CD8α-PE, CD40-PE, CD31-PECy7, CD11b-PeCy5/-PerCPCy5.5/-PECy7, CD45R(B220)-FITC/-PE/-PECy7, CD11c-PETxRed/-APC/-APCCy7, MHC class II-PE, F4/80-FITC/-APCCy7/-APC, CD16/32-AF700, CD45-PacificBlue, CD206-APC were purchased from eBioscience. Polyclonal anti-CTCF-biotin antibody (antibodies-online.com) was used for intracellular detection of CTCF protein. Biotinylated antibodies were detected by streptavidin-PacificBlue (eBioscience).

In freshly isolated spleen, peritoneal wash or liver cells, leukocytes were defined as follows: lymphocytes (CD11b-CD11c- and B220+, CD4+ or CD8+), neutrophils ($SSC^{lo}CD11b^{hi}$-$Ly6G^{+}$), monocytes ($SSC^{lo}CD11b^{hi}Ly6G^{-}Ly\text{-}6C^{+}$), myeloid DC ($B220^{-}CD11c^{hi}$) and macrophages ($CD11c^{low}CD11b^{hi}F4/80^{+}$). Detection of the LacZ reporter was performed using fluorescein-di-β-D-galactopyranoside substrate (FDG; Invitrogen) as previously described (25). Samples were acquired on LSR-II or Calibur (BD Bioscience) and analyzed using FlowJo software (TreeStar).

Cell cultures

In vitro differentiation of bone marrow cells into macrophages was performed using 10% L929-cell culture medium (conditioned-medium), as previously described (26). Briefly, bone marrow cells were isolated and seeded in a petridish (Sarstedt) at $0.5x10^6$ cells/ml, in a volume of 8 ml. At day 4, 10 ml conditioned-medium was added. On day 7, adherent cells were harvested. Purity of the $F4/80^{+}CD11b^{+}$ cells was always > 85%. Next, $0.5x10^6$/ml bone marrow derived macrophages were stimulated with LPS (100 ng/ml, from *S. Minnesota*, Invivogen or *E. Coli* 026:B6, Sigma), R848 (1 µg/ml, Alexis) or CpG-1668 (5 µg/ml, Invivogen). Following overnight incubation, supernatants were harvested and measured by ELISA for IL-10, IL-6, TNF and IL-12p40 (eBioscience) according to manufacturers' protocol.

Multilamellar liposomes labeled with DiI in the aqueous phase were prepared as described previously (27, 28). Liposomes consisted of phosphatidyl choline and cholesterol in a 6:1 molar ratio. After washing, the liposomes were resuspended in phosphate-buffered saline (PBS). For the

in vitro phagocytosis test, DiI-liposomes (1%) were added to the macrophage cultures, and labeled cells were detected using FACSCalibur.

Protein analysis by western blotting

For Western blotting, macrophages were lysed with 2x Laemmli buffer (whole cell extracts), and nuclear extracts of cultured cells were isolated as described before (29). Polyclonal anti-CTCF antibody (Bioke) and anti-RCC1 (Santa Cruz) was incubated overnight at 4°C in Tris-buffered saline containing 5% BSA and 0.15% (v/v) NP-40. Blots were incubated with secondary goat anti-rabbit antibody coupled to horseradish peroxidase (GE Healthcare UK Ltd: 1:50,000). Signal detection was performed using ECL (Amersham).

Immunohistochemistry

Liver was fixed in 4% formaldehyde or snap-frozen after removal. Tissue was embedded in paraffin or TissueTek and fixed with cold acetone for 2 min. For paraffin embedded tissue, F4/80 and CTCF antigens were retrieved by proteinase K and TE buffer, respectively. Endogenous peroxidase activity was removed by 20 min incubation with 0.3% H_2O_2. Tissue sections were further blocked with 10% rabbit serum and 5% BSA in PBS, 0.1% avidin and 0.01% biotin (DAKO) consecutively for 15 min for each blocking step. Next, tissue sections were incubated with the primary antibody (F4/80, CTCF or MHC II), with or without biotin conjugated-rabbit-anti-rat Ig (DAKO) and streptavidin HRP (DAKO) or goat-anti-rabbit HRP for 1 hour with proper washing after each step. The staining was visualized using diaminobenzidine (DAB, Invitrogen), and counterstained with hematoxyline (Sigma). Digital images of 4 randomly selected high power fields (20x magnification) were captured using NIS-Elements D 3.0 software (Nikon Digital Sight DS-U1). The average of the number of MHC-II and F4/80 positive cells from 4 high power field was determined and expression of MHC-II was graded as 1 (<20 positive cells) until 4 (>120 positive cells).

Isolation of RNA, generation of cDNA, quantitative PCR and gene expression analysis

RNA was isolated using the Total RNA purification kit (Ambion, Life Technologies) or NucleoSpin RNAII kit (Bioké) as described in the manufacturer's protocol. The quantity and quality of RNA were determined using a NanoDrop spectrometer (NanoDrop Technologies). Total RNA (0.5–1.0 µg) was used as a template for cDNA synthesis by iScript cDNA Synthesis Kit (Bio-Rad Laboratories) or Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. Quantitative real-time PCR was performed using the Bio-Rad optical 96-well plates with a MyIQ5 detection system (Bio-rad Laboratories) or ABI Prism 7700 sequence detection system

(Applied Biosystems). The probe in the master mix (TaqMan® Gene Expression Master Mix) was an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye. Primers for housekeeping gene 18S (Hs99999901_s1), TLR4 (Mm00445274_m1), TLR8 (Mm01157262_m1) and TLR9 (Mm00446193_m1) were purchased from Applied Biosystems. The nucleotide sequences of other primers used are listed in Supplementary Table S1. The expression of genes was normalized to 18S or GAPDH.

For microarray gene expression analysis, labeling and hybridization with GeneChip Mouse-Gene 1.0 ST arrays was performed according to the manufacturer's protocol and scanned with Affymetrix GeneChip Command Console software. In total, 8 arrays were analyzed (5 CTCF-KO and 3 wild type samples from three independent experiments). Data was filtered using a multistep filtration method, which involves the application of receiver operating characteristic analysis for the estimation of cut-off signal intensity values. Only probe set identifiers (IDs) having gene assignments (annotation date: 21 July 2008; Affymetrix) were used for analysis. A relative gene expression value was calculated by normalization to the median expression value for the gene across samples. Efron-Tibshirani's test uses 'maxmean' statistics to identify gene sets differentially expressed. The threshold of determining significant gene sets was set to 0.005.

Data analysis and statistics

For all experiments, the difference between groups was calculated using the Mann-Whitney U test or Wilcoxon T test for unpaired data (GraphPad Prism version 4.0; GraphPad Software). Differences were considered significant when $P < 0.05$. Results are presented as the mean ± SEM, unless otherwise indicated.

Results

Deletion of Ctcf gene in the macrophage subpopulations

To examine the role of Ctcf in the myeloid cell lineage *in vivo*, we crossed mice carrying a *Ctcf*-floxed allele (18, 23) with *LysM-Cre* transgenic mice, which express the *Cre* recombinase under the control of the LysM promoter (18, 23), thereby confining *Ctcf* gene deletion to myeloid cells.

First, we confirmed that the *LysM-Cre* transgene is functionally expressed in various macrophage populations, using a mouse Cre-reporter strain harboring a targeted insertion of enhanced yellow fluoresencent protein (EYFP) into the ROSA26 locus (24). We found substantial EYFP expression in $CD11b^{+}F4/80^{high}$ peritoneal and splenic macrophages, as well as in $CD11b^{+}F4/80^{high}$ Kupffer cells in the liver, although in all of these compartments EYFP-negative cells were also present (Figure 1A).

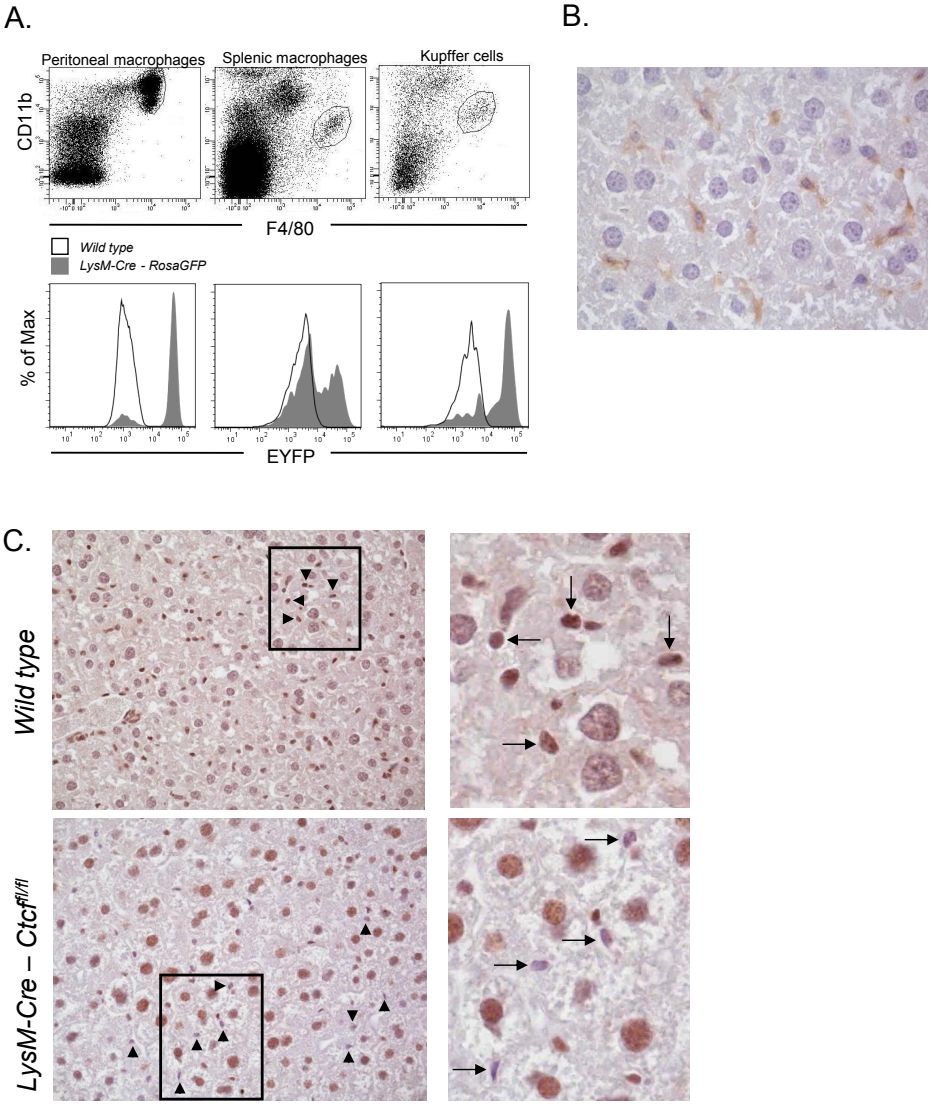

Figure 1. LysM promoter is active in macrophages and drives *Ctcf* deletion in macrophages in *LysM-Cre Ctcf$^{f/f}$* mice.

(A) Representative flow cytometric plot and histogram to visualize the activity of LysM promoter in LysM-Cre Rosa-EYFP mice. Peritoneal and splenic macrophages, and Kupffer cells were identified as CD11c^{low} CD11b^{+} F4/80^{+} cells. EYFP expression results from LysM-driven Cre-recombinase deletion of "floxed-stop" fragment upstream EYFP. Flow cytometric analysis shows that ~80%, ~35% and ~70% of peritoneal and splenic macrophages, and Kupffer cells, respectively, are EYFP+, indicative for LysM activity in these macrophage populations. (B) Representative F4/80 and hematoxyline stainings of the liver of wild type animals. The nuclei of hepatocytes are characterized by their large size and round shape. Additionally, small and elongated nuclei, of which ~70% are associated with F4/80 expression, are observed. (C) Representative nuclear CTCF and hematoxyline stainings of the livers of wild-type and *LysM-Cre Ctcf$^{f/f}$* animals. Nuclear expression of Ctcf, observed as brown staining, is weaker in the non-hepatocyte cells of *LysM-Cre Ctcf$^{f/f}$* compared to the wild-type animals.

Homozygous *LysM-Cre Ctcf*$^{f/f}$ mice appeared normal and were fertile and born at the expected frequencies on the basis of Mendelian inheritance. Deletion of the *Ctcf* gene was monitored by the expression of the bacterial β-galactosidase (*lacZ*) reporter present in the floxed *Ctcf* allele (18). As expected (23), we found *lacZ* expression, detected by fluorescein-di-β-D-galactopyranoside in conjunction with cell-specific surface markers, in substantial fractions of myeloid cell populations, including granulocytes, monocytes and macrophages of *LysM-Cre Ctcf*$^{f/f}$ mice (not shown). To assess whether deletion of the *Ctcf* allele resulted in the lack of Ctcf protein expression, we performed immunohistochemical analyses in the liver. Kupffer cells can be identified by expression of the F4/80 markers and differ from hepatocytes present in the liver by their smaller and more elongated cell nucleus (Figure 1B). When we analyzed expression of Ctcf, we noticed that Kupffer cells manifested a dense nuclear staining, whereas hepatocytes show a less intense nuclear staining (Figure 1C). Immuno-histochemical analysis of liver specimens from *LysM-Cre Ctcf*$^{f/f}$ mice demonstrated that Kuffper cells were still present in apparently normal frequencies. In a large fraction of Kupffer cells the expression of Ctcf was lost, although also Ctcf-expressing Kupffer cells were detected (Figure 1C).

To assess whether deletion of *Ctcf* influenced the size of the macrophage compartment, we used flow cytometry to compare the proportions of individual myeloid subpopulations in peritoneum and spleen. In the peritoneal cavity of *LysM-Cre Ctcf*$^{f/f}$ mice the proportions of macrophages were moderately reduced and the proportions of lymphocytes and myeloid DC were increased, compared with wild-type controls (Suppl. Figure 1A). In the spleen of *LysM-Cre Ctcf*$^{f/f}$ mice, we observed a reduced frequency of monocytes, but there were no significant differences in frequencies of macrophages, when compared with wild-type controls (Suppl. Figure 1B).

Taken together, although *LysM*-promoter mediated *Cre* expression resulted in deletion of the *Ctcf* gene in a substantial proportion of macrophages, Ctcf-defiency had only moderate effects on the frequencies of these cell populations in peritoneum, spleen and liver of *LysM-Cre Ctcf*$^{f/f}$ mice.

Reduced MHC class II expression in the liver of LysM-Cre Ctcf$^{f/f}$ *mice*

Previous studies indicated that Ctcf plays an important role in regulation of MHC class II expression in human B cells (30). Despite significant deletion of Ctcf (Figure 1), flow-cytometric analyses of peritoneal or splenic macrophages did not show evidence for reduced surface MHC class II expression in *LysM-Cre Ctcf*$^{f/f}$ mice, compared with wild-type littermates (Figure 2A). In contrast, flow-cytometric and histological analysis of the liver of *LysM-Cre Ctcf*$^{f/f}$ mice demonstrated substantial reduction of MHC class II expression (Figure 2A and 2B). Quantification of MHC class II

expression in histological samples of the liver showed a highly significant reduction in gene-targeted mice, compared with the control mice (Figure 2C, $p<0.0001$). Since the majority of MHC class II-expressing cells in the liver are F4/80$^+$ Kupffer cells, we assessed their numbers and observed that the proportion of F4/80-expressing cells was not affected (Figure 2B and 2C), demonstrating that deletion of Ctcf leads to lower levels of expression of MHC class II and not to deletion of MHC class II-expressing Kupffer cells.

These findings indicate that Ctcf plays an important role in the regulation of MHC class II expression in Kupffer cells, but not in the other macrophage populations analysed.

Reduced in vitro macrophage differentiation from LysM-Cre Ctcf$^{f/f}$ bone marrow

To study the effects of Ctcf on macrophage activation and function, we generated macrophages by *in vitro* differentiation from BM precursors using L-929 conditioned medium (Figure 3A). The yield of CD11b$^+$F4/80$^+$ macrophages from total BM of *LysM-Cre Ctcf$^{f/f}$* mice generated after 7 days of culture was significantly reduced compared with wild-type littermates mice ($p<0.05$; Figure 3B). Analysis of *Ctcf* mRNA expression by quantitative RT-PCR showed a reduction to ~33% of wild-type levels (n=6), which was also reflected by substantial reduction of Ctcf protein as analyzed in western blotting experiments (Supplementary Figure S2). Since we observed efficient Ctcf deletion by *lacZ* expression in mature macrophage populations *in vivo* (not shown), these findings point to a long half-life of Ctcf protein or a specific survival of Ctcf-expressing cells.

LysM-Cre Ctcf$^{f/f}$ and wild-type macrophages did not differ in surface expression of lineage-associated markers F4/80 and CD11b (Figure 3A) or the activation markers CD86 and MHC class II (Figure 3C and 3D). We observed lower CD206 and CD16/32 expression on *LysM-Cre Ctcf$^{f/f}$* than on wild type macrophages, but these differences were not statistically significant. However, surface CD40 expression was significantly reduced on *LysM-Cre Ctcf$^{f/f}$* macrophages, when compared with wild-type macrophages, both unstimulated and upon LPS stimulation (Figure 3C and 3D).

We found that BM-derived macrophages from *LysM-Cre Ctcf$^{f/f}$* mice and wild-type mice had a similar capacity to phagocytose DiI-labeled liposomes (Figure 3E). Finally, the viability of *LysM-Cre Ctcf$^{f/f}$* and wild-type macrophages was similar, both with and without LPS stimulation (not shown).

Therefore, the absence of Ctcf was associated with reduced *in vitro* differentiation of BM-derived macrophages, both in cell numbers and in terms of surface CD40 expression.

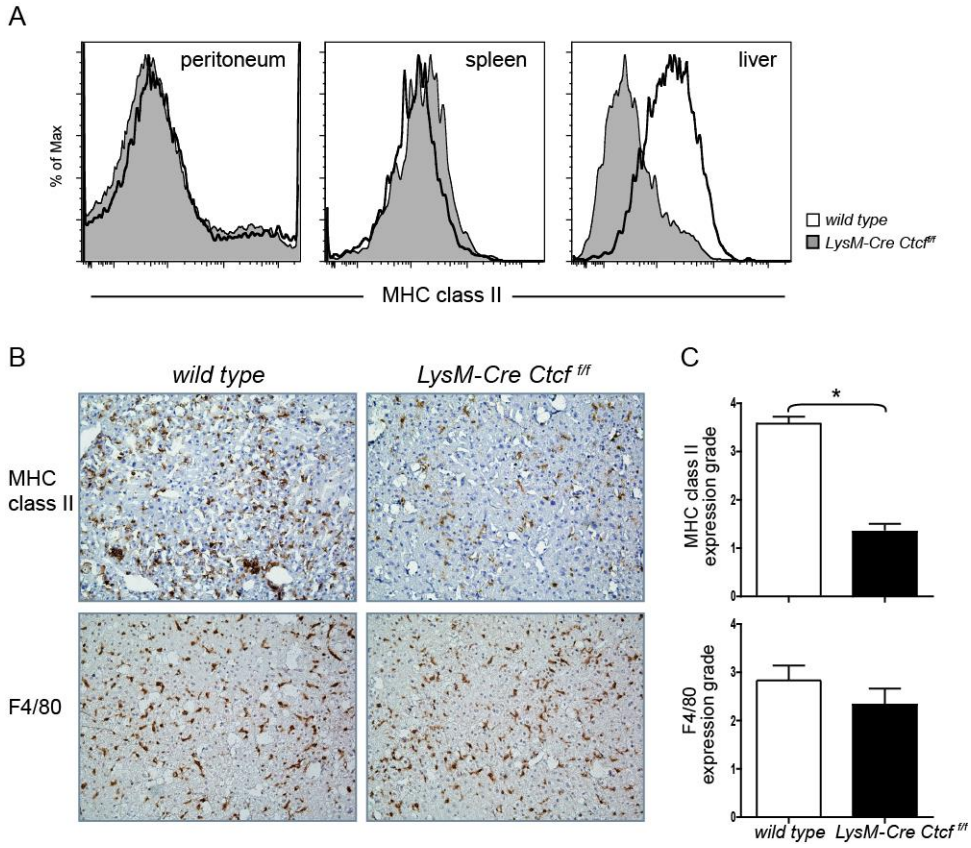

Figure 2. *Ctcf* deletion results in lower MHC class II expression on Kupffer cells in the liver.
(A) Representative histograms showing MHC class II expression by macrophages in peripheral organs. Grey histograms represent *LysM-Cre Ctcf*$^{f/f}$ and black-line histograms represent wild type macrophages. Total non-parenchymal cells from the liver were isolated as described in Materials and Methods. Macrophages in the liver were identified as CD45^{+} CD11c^{low} CD11b^{+} F4/80^{high}. The staining was performed on at least 5 wild type and 5 *LysM-Cre Ctcf*$^{f/f}$ mice with similar results. (B) Representative immunohistochemical staining for MHC class II and F4/80 on liver tissue sections from wild type and *LysM-Cre Ctcf*$^{f/f}$ mice. MHC class II expression (upper panel) of *LysM-Cre Ctcf*$^{f/f}$ mice was lower than in wild type mice while the expression of F4/80^{+} cells was similar (lower panel). The staining is representative of 9 wild type and 9 *LysM-Cre Ctcf*$^{f/f}$ mice. (C) Quantitative analysis of MHC class II and F4/80 expression. Liver tissue sections from 9 *LysM-Cre Ctcf*$^{f/f}$ and 9 wild type mice were scored (1 to 4) for the degree of MHC class II and F4/80 positive staining. The grading for MHC class II positivity, but not for F4/80, in the livers of *LysM-Cre Ctcf*$^{f/f}$ mice was significantly lower than in wild type livers ($p<0.0001$).

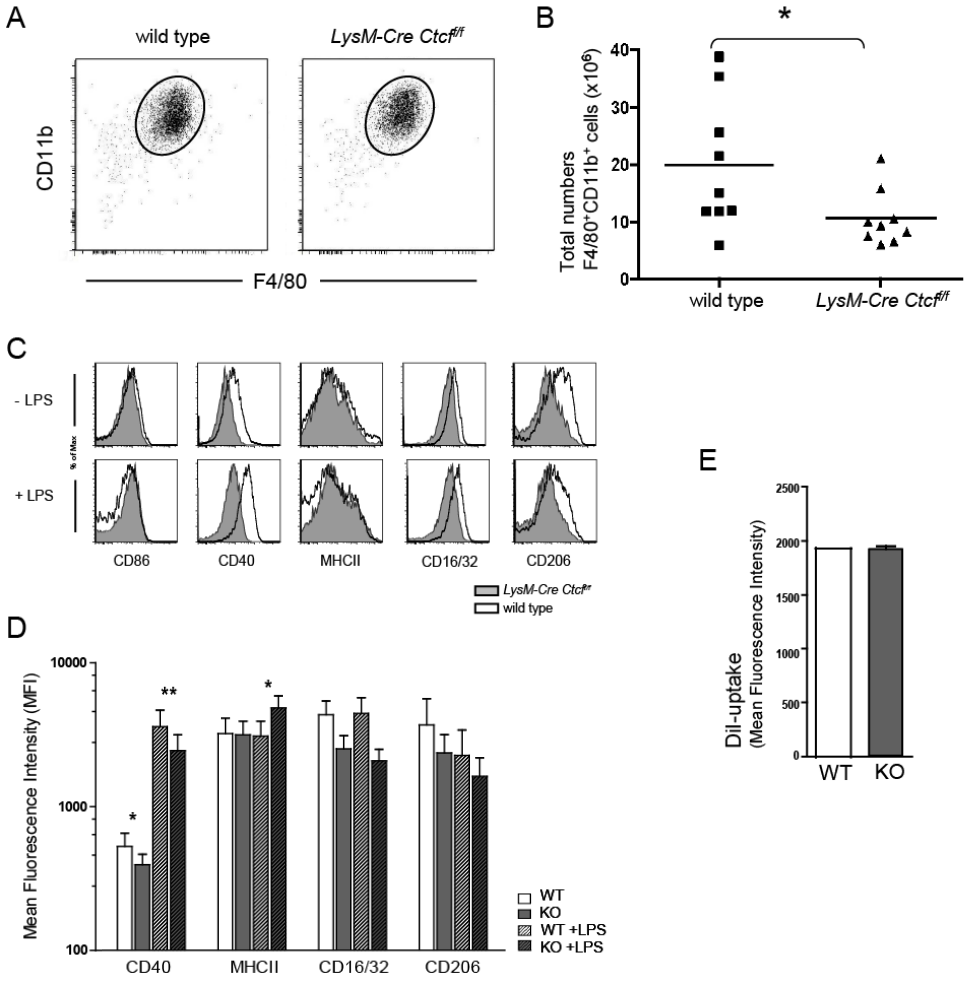

Figure 3. *Ctcf* deletion impairs *in vitro* macrophage differentiation from bone marrow cells
(A) FACS plots that define bone marrow derived macrophages as CD11b^{+}F4/80^{+} cells. Cultured macrophages from *LysM-Cre Ctcf$^{f/f}$* mice express similar levels of CD11b and F4/80 as wild type macrophages. The assay was performed with cells from 9 wild type and 9 *LysM-Cre Ctcf$^{f/f}$* mice with similar results. (B) Macrophages were generated *in vitro* as described in Materials and Methods and the cell yields were determined at the end of the culture (day 7). The number of macrophages derived from *LysM-Cre Ctcf$^{f/f}$* mice was significantly lower than from wild type mice (p=0.0235). (C) Representative histograms of CD86, CD40, MHC class II, CD206 and CD16/32 expression by cultured macrophages from wild type (black line histogram) and *LysM-Cre Ctcf$^{f/f}$* mice (grey histogram) unstimulated (upper row) or after LPS stimulation (lower row). (D) Average mean fluorescence intensity (MFI) expression of analyzed molecules in wild type (WT) and *LysM-Cre Ctcf$^{f/f}$* (KO) macrophages before or after stimulation with LPS. Data are presented as average MFI ± SEM from 7 wild type and 10 *LysM-Cre Ctcf$^{f/f}$* mice. *p<0.05 and **p<0.01 (E) Phagocytosis capacity of cultured macrophages was measured by incubating them overnight DiI-liposomes. Uptake of liposomes was quantified by flow cytometry. Data represent MFI ± SD from two independent cultures from each genotype.

LysM-Cre Ctcf$^{f/f}$ macrophages exhibit reduced TLR-induced IL-10 and TNF production

Next, we analyzed the ability of *LysM-Cre Ctcf$^{f/f}$* macrophages to produce cytokines upon induction by different TLR agonists. Upon stimulation with LPS and R848, *LysM-Cre Ctcf$^{f/f}$* macrophages produce lower levels of IL-10 than did wild type macrophages ($p<0.05$), whereas upon CpG stimulation differences in IL-10 production were not significant (Figure 4). Stimulation with all 3 distinct TLR agonists showed a significant reduction of TNF production by macrophages from *LysM-Cre Ctcf$^{f/f}$* mice, when compared with controls ($p<0.05$). In contrast, the production of IL-12p40 or IL-6 by *LysM-Cre Ctcf$^{f/f}$* or wild-type macrophages was similar upon TLR ligation (Figure 4).

The IL-10 family of cytokines includes IL-19, IL-20 and IL-24. The genes encoding these cytokines are all located in a cluster together with the IL-10 gene on chromosome 1q31-32 (31, 32) (Figure 5A). In parallel to the Th2 cytokine locus (19), we hypothesized that also the IL-10 locus may contain several Ctcf sites, which would enable long-range chromatin interactions between regulatory elements and promoter regions for the individual cytokine genes. Since CTCF-binding sites are generally common to different cell types (33, 34), we made use of our reported dataset of CTCF-binding sites identified in cultured primary pre-B cells by chromatin immuno-precipitation coupled to high-throughput sequencing (ChIP-Seq) (22). We found that the IL-10 locus contains several Ctcf binding sites, which are not located in the promoter regions of individual cytokine genes, but rather between the cytokine genes (Figure 5A). This would be consistent with a role for Ctcf in looping and enabling long-range DNA interactions that are essential for coordinated expression of the individual genes in the IL-10 locus.

We therefore set out to determine whether besides IL-10 protein production, also transcription levels of the *Il10* gene and the closely linked *Il19, Il20* and *Il24* genes were reduced in *LysM-Cre Ctcf$^{f/f}$* macrophages upon TLR ligation. In line with the observation at the protein level, IL-10 mRNA expression was reduced in *LysM-Cre Ctcf$^{f/f}$* macrophages, particularly upon R848 stimulation ($p<0.05$; Figure 5B). Interestingly, *LysM-Cre Ctcf$^{f/f}$* macrophages exhibit lower expression of the other three IL-10 locus cytokines as well, whereby significance was reached for IL-19 upon R848 stimulation ($p<0.05$) and for IL-20 upon LPS or CpG stimulation ($p<0.05$; Figure 5B). The observed reduced cytokine production in Ctcf-deficient macrophages cannot easily be explained by an effect of Ctcf on TLR expression, as LysM-Cre Ctcf$^{f/f}$ BM-derived macrophages did not manifest reduced levels of Tlr4, Tlr8 or Tlr9 mRNA (Supplementary Figure S3).

Taken together, these data show that the absence of Ctcf in macrophages does not appear to affect their capacity to produce IL-6 or IL-12, but is associated with reduced expression of TNFα and IL-10 locus cytokines upon stimulation with various TLR ligands.

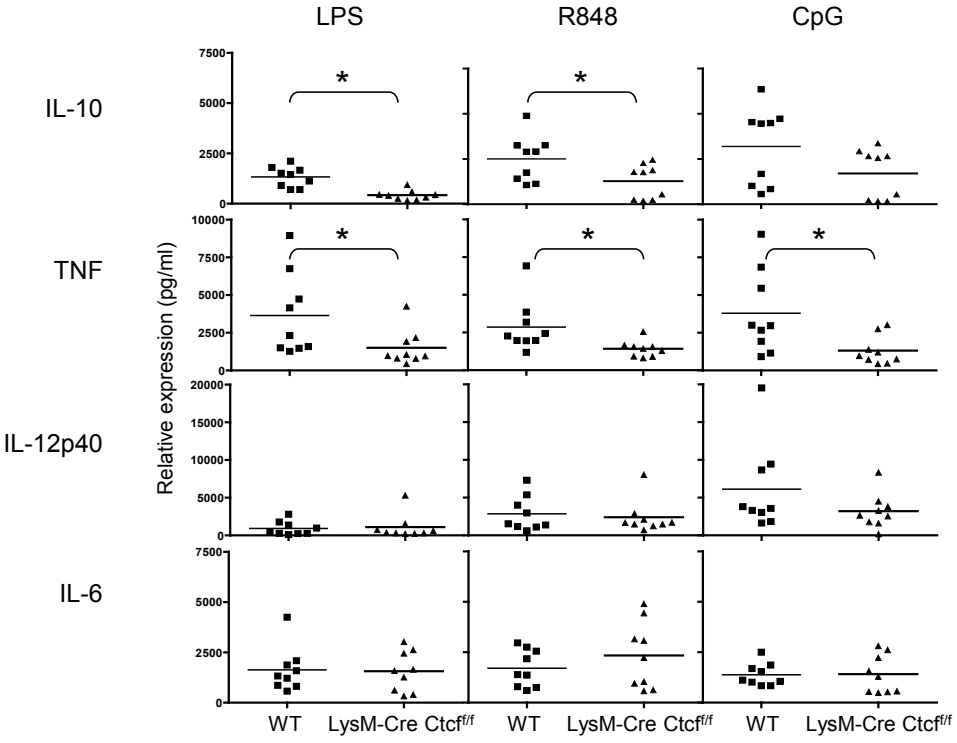

Figure 4. Impaired cytokine induction by bone marrow derived macrophages from *LysM-Cre Ctcf$^{f/f}$* mice upon TLR4, TLR7/8 and TLR9 ligation

1x10^5 bone marrow derived macrophages from wild type and *LysM-Cre Ctcf$^{f/f}$* mice were stimulated with LPS, R848 and CpG for 24h. The levels of IL-10, TNF, IL-12p40 and IL-6 in supernatant were measured by ELISA. Data show concentrations of produced cytokines (pg/ml). The experiments were performed using independent cultures from 9 wild type and 9 *LysM-Cre Ctcf$^{f/f}$* mice.

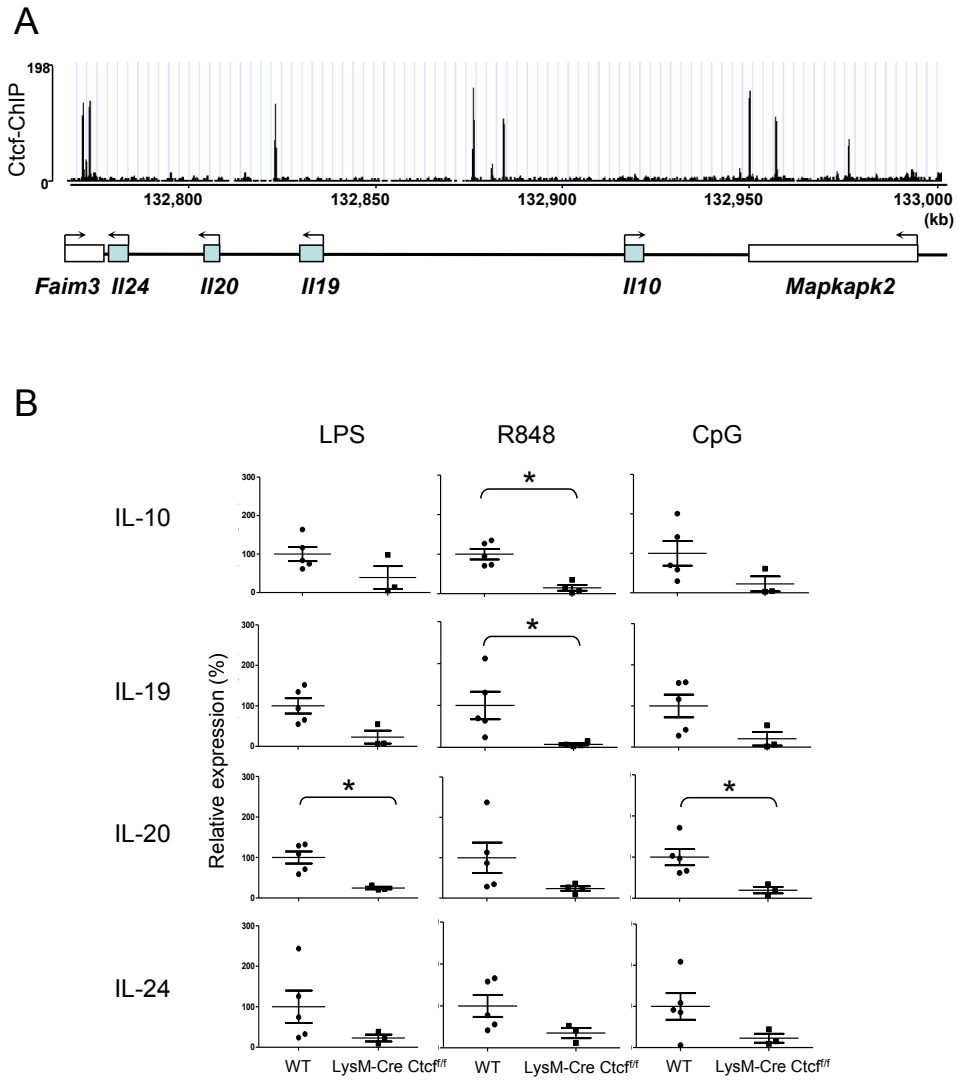

Figure 5. Impaired TLR-induced mRNA expression of IL-10 family members in bone marrow derived macrophages from *LysM-Cre Ctcf$^{f/f}$* mice

(A) Mouse genomic region containing the IL-10 gene family locus. CTCF ChIP-Seq data is depicted above the localization of the IL-10 family (IL-10, IL-19, IL-20 and IL-24) and flanking genes. ChIP-Seq data were obtained from cultured pre-B cells [21-22]. kb= kilobases.

(B) Bone marrow derived macrophages from wild type and *LysM-Cre Ctcf$^{f/f}$* mice were stimulated for 5h with LPS, R848 or CpG. IL-10, IL-19, IL-20 and IL-24 mRNA expression in macrophages were quantified by real time PCR, and expressed relative to GAPDH. The values depicted show of 3-5 mice per experimental group. Statistical analysis: unpaired T-test, * denotes P<0.05.

Gene expression profiling in BM-derived LysM-Cre Ctcf$^{f/f}$ macrophages upon LPS stimulation

Finally, we aimed to investigate the effect of *Ctcf* deletion in macrophages in a genome-wide fashion. To this end, we performed gene expression profiling of *LysM-Cre Ctcf$^{f/f}$* and wild type macrophages upon overnight LPS stimulation. Of 23,500 detected genes, 617 genes (212 up and 405 down) were differentially expressed between *LysM-Cre Ctcf$^{f/f}$* and wild-type macrophages (threshold of two-fold). The 100 most downregulated and 100 most upregulated genes are shown in Table 1. As expected, *Ctcf* was among these most downregulated genes. We analyzed the expression of genes that were downregulated in Ctcf-deficient macrophages as compared to wild type macrophages. These downregulated genes included CCL8 and CCL12 (also known as monocyte chemotactic protein (MCP)-2 and MCP-5, and Cxcl10 (also known as IP-10), which are chemotactic for and activate numerous immune cells, such as monocytes, T cells and NK cells. In addition, genes involved in antibacterial (Nos2) or antiviral responses (Mx1, Mx2, ISG20, and Rsad2) were strongly downregulated, suggesting weaker responses to eliminate pathogens. Furthermore, we observed reduced expression of molecules known to negatively regulate immunity, including IL1rn, cd274 (PD-1 ligand), fgl-2 (fibrinogen-like protein 2), which may have the same functional consequence as the lower levels of the immunosuppressive cytokine IL-10 as we observed following stimulation with LPS or R848. We also found reduced expression of the nitric oxide synthetase Nos2, which catalyzes the production of nitric oxide and contributes to the anti-microbial or anti-tumor function of macrophages as part of the oxidative burst.

Many of the genes that were significant upregulated in Ctcf-deficient macrophages, including meiotic nuclear divisions 1 homologue (Mnd1, ~14x), the CD69 antigen (~13x) and Integral membrane protein 2a (Itm2a, ~9x), were previously found to be controled by Ctcf in precursor B cells (Mnd1, CD69, Itm2a) (22) or during mammalian limb development (Mnd1, Itm2a) (35) and may reflect direct cell-lineage independent targets of Ctcf. Other genes are expected to be more indirectly regulated by Ctcf, e.g. the sugar transport facilitator Slc2a3 (~11,5x), perhaps through crosstalk between the Ctcf-regulated imprinted growth demand gene Igf2 (36). Among genes upregulated in Ctcf-deficient macrophages, were also genes with a more macrophage-specific function, including the 5-lipoxygenase (Alox5, ~7x) enzyme that is involved in the generation of leukotriens that enhance phagocytosis and NADPH oxidase Nox1 (~5x), an important source of reactive oxygen species in macrophages. In addition, Ctcf-deficient macrophages expressed e.g. higher levels of the chemokine receptor CX3CR1 (~3x), which is known to regulate intestinal macrophage homeostasis (37) and phosphodiesterase Pde2a (~3x), which is induced during M-CSF differentiation of macrophages (38).

Table 1. Genes modulated in CTCF-deficient macrophages.

Genes Downregulated in CTCF-deficient macrophages	
Gene	**Ratio**
Lipg	0,072
Ccl8*)	0,073
Htr2b	0,130
Nos2	0,136
Iqgap2	0,156
C1rb	0,161
Gbp5	0,167
Dhfr	0,168
Ctcf	0,178
Ednrb	0,179
Thbs1	0,183
Cd300lf	0,184
Slfn1	0,189
Mertk	0,195
Fgl2	0,201
Mmp27	0,205
Dcn	0,206
Slc40a1	0,213
Lrrc14b	0,214
Trib3	0,215
Ch25h	0,223
Kcnab1	0,226
Slc28a2	0,229
Ccrl2	0,229
Vegfa	0,229
Tspan13	0,231
Soat2	0,239
Slco3a1	0,242
Gprc5b	0,243
Ifitm6	0,244
Ddit3	0,248
Gbp2	0,249
Tmem140	0,249
Tmod1	0,253
Gadd45b	0,253
Cd5l	0,254
Hal	0,256
Klra3	0,257
Rhov	0,260
Gtpbp2	0,261
Hyal1	0,262
Gadd45a	0,264
Sp140	0,264
Mx1	0,265
Tlr6	0,266
Mx2	0,266

Gene	Ratio
Tgm2	0,267
Rsad2	0,267
Msh3	0,269
Cmpk2	0,269
Cxcl11	0,270
Phf11	0,271
Serpinb1c	0,277
Timp1	0,280
Ctsk	0,287
Fabp5	0,292
Rhoc	0,294
Ifi205	0,297
Ppap2b	0,299
Treml2	0,299
Cd274	0,300
Csprs	0,302
Plk2	0,308
Ccdc99	0,311
Nupr1	0,312
Tnfrsf26	0,313
Nr1d2	0,313
Csf3r	0,316
E430029J22Rik	0,320
Gbp6	0,320
Sectm1a	0,320
Tgtp	0,320
Mxd1	0,321
Stoml1	0,322
Il1rn	0,322
Gbp1	0,323
Slamf7	0,324
Igsf9	0,326
Ccl12	0,326
Nt5c3	0,331
Mthfd2	0,331
EG634650	0,334
Mpa2l	0,335
Cxcl10	0,337
Olfr1444	0,339
Isg20	0,342
Clec2d	0,342
Iigp1	0,343
Plac8	0,343
Tsc22d3	0,344
Tmem26	0,346
Carhsp1	0,347
S100a1	0,349
Gbp3	0,353
Sell	0,354

Genes Upregulated in CTCF-deficient macrophages	
Gene	**Ratio**
Uchl1	16,58
Mnd1	13,71
Cd69	12,77
Slc2a3	11,50
Itm2a	9,22
Dner	8,96
Fam171b	7,50
Alox5	7,28
Sqle	7,21
Clec4b1	6,80
Opn3	6,52
Ptrf	6,11
Prps2	5,75
Asrgl1	5,53
Emb	5,07
Nox1	5,05
Slc25a4	4,96
Slc35e3	4,95
Glt25d2	4,94
Ppbp	4,81
C1s	4,77
Anp32e	4,71
Clec4n	4,62
Vcan	4,58
Htra1	4,39
L1cam	4,25
Gpr183	4,23
Clec2i	4,22
Gbgt1	4,11
Fads3	4,07
Uck2	4,03
Zcwpw1	3,86
Nme4	3,73
Gcsh	3,72
Rasgrp3	3,69
Myc	3,68
Lifr	3,67
Nudt15	3,67
Fads2	3,63
Scd1	3,62
Emr4	3,58
Vcl	3,51
Ebpl	3,51
Grk4	3,51
Cd109	3,49
Pf4	3,49

Gene	Ratio
Scin	3,48
Rragb	3,45
Idi1	3,42
Cldn12	3,41
Clec4a2	3,40
Ptpla	3,39
Ephx1	3,37
Klhl6	3,36
Fbxl21	3,34
Dusp6	3,34
Fh1	3,32
Ccr1	3,32
Pgm2l1	3,32
Apol7c	3,31
H2-M2	3,29
Tfrc	3,27
Clec7a	3,26
Ppap2a	3,22
Zswim7	3,21
Padi2	3,21
Ahi1	3,20
Ccl22	3,20
Gmds	3,20
Fscn1	3,20
Gprc5c	3,19
Rgs18	3,18
Zdhhc2	3,16
Prune2	3,16
Prkar2b	3,14
Nme1	3,14
Ccna2	3,12
Gria3	3,10
Cep78	3,10
Adrb2	3,07
Mthfd1	3,06
Acot7	3,06
Tomm20	3,06
Igf2bp3	3,05
Rhof	3,01
Dock1	3,00
Pik3cg	3,00
Paf1	2,99
Lpl	2,98
Mettl1	2,98
Fchsd2	2,97
Gnai1	2,97
Pde2a	2,97
Cx3cr1	2,95
Plaur	2,93

*) shaded genes are discussed in the text.

Discussion

Ctcf has been identified as an important regulator of long-range chromatin interactions in lymphocytes (4), but the role of Ctcf in macrophages cells has not been investigated. Macrophages are crucial cells in the immune responses to bacteria and viruses, and to remove apoptotic cells or cellular debris. Triggering of macrophages via a broad array of pattern recognition receptors is the initiating step, and this quickly leads to uptake of the pathogen and debris, and the release of effector molecules, such as cytokines. This study describes for the first time the effect of specific Ctcf deletion in macrophages in mice. *In vivo*, deletion of Ctcf resulted in a mild phenotype. Furthermore, there was a strongly reduced expression of MHC class II in the liver of *LysM-Cre Ctcf$^{f/f}$* mice, and to a lesser extent in the spleen. Finally, macrophages generated *in vitro* from bone marrow of *LysM-Cre Ctcf$^{f/f}$* mice showed a normal phenotype, but a significantly reduced capacity to induce the expression of IL-10 family genes, and the production of IL-10 and TNF upon stimulation with different TLR ligands, while IL-12p40 and IL-6 levels were not affected.

The importance of Ctcf in controlling MHC class II expression has been reported before (30, 39). In line with this, we found specific reduction of MHC class II expression on Kupffer cells in the liver in *LysM-Cre Ctcf$^{f/f}$* mice. Since the number of Kupffer cells in the liver was not affected, our findings corroborate the previously established participation of Ctcf in expression of genes within the MHC locus. However, we did not observe a change of the MHC class II expression in splenic, peritoneal or in cultured macrophages. Interestingly, three genes located within the MHC locus *(Cfb, Daxx* and *Tap2)* were downregulated in LPS stimulated *LysM-Cre Ctcf$^{f/f}$* versus wild type macrophages (ratio: 0.49; 0.43; 0.49, respectively), suggesting that the absence of modulation of MHC class II expression could reflect defective long-range interactions in the MHC locus in the absence of Ctcf in Kuppfer cells in mice. In addition, differential expression levels of LysM in distinct tissues may also cause the differences in the observed phenotype. Reduced numbers of peritoneal and cultured macrophages as we observed in *LysM-Cre Ctcf$^{f/f}$* mice may be the result of upregulation of the proto-oncogene c-Myc compared to controls, as we observed in our microarray analysis (~3.6x; Supplementary Table S1), which is also in line with previously reported findings (40). Overexpression of c-Myc may result in enhanced proliferation but may also induce apoptosis. Next to c-Myc, other differentially expressed genes in *LysM-Cre Ctcf$^{f/f}$* macrophages were significantly represented in cell cycle, cell death and proliferation networks, and may consequently modulate macrophage numbers. Myeloid cells downregulate Ctcf expression during maturation (41) and overexpression of Ctcf in myeloid progenitors resulted in strongly impaired development and survival of DC (17), suggesting that a fine regulation of Ctcf expression may be important for competent myeloid cell

development.

A set of genes downregulated during macrophage differentiation but induced upon TLR stimulation are the Schlafen (Slfn) genes, especially *Slfn4* (42). In our data set, the expression levels of *Slfn1*, *Slfn4* and *Slfn9* genes were lower after LPS stimulation in *LysM-Cre Ctcf$^{f/f}$* than in control macrophages (ratio 0.21; 0.38; 0.38, respectively). Since we showed that the deletion of Ctcf does not significantly affect the expression levels of TLR4, TLR8 and TLR9 mRNA, and since these genes are not direct targets of Ctcf, the observed effect may have been indirectly caused through type I IFN (42). Regulation of TNF expression by Ctcf is in agreement with the literature (43). Additionally, we show diminished IL-10 production by macrophages as a result of Ctcf depletion, similarly to low IL-10 production upon Ctcf deletion in Th2 cells as we previously observed (19). In macrophages, TLR4 stimulation induces IL-10 production through TRAF3 and NFkB (26, 44) and –indeed- we found downregulation of genes downstream of the NFkB complex in *LysM-Cre Ctcf$^{f/f}$* macrophages. We found that the IL-10 gene harbors Ctcf-binding sites on both the 5' and 3' end in pre-B cells. These data demonstrate that Ctcf binding sites are present in the complex IL-10 cytokine locus, although it is unclear whether Ctcf binds to this locus in macrophage. Nevertheless, since Ctcf sites are relatively invariant across diverse cell types and since Ctcf has an essential role in chromatin architecture, one may appreciate the possible importance of Ctcf in the reorganization of the IL-10 locus that occurs upon TLR stimulation (45). In line with this, our data demonstrate that reduced Ctcf expression in macrophages modulated the expression of the IL-10 homologues IL-19, IL-20 and IL-24, which are expressed within a highly conserved cytokine gene cluster. Our findings identify Ctcf as an important regulator of the IL-10 family gene locus.

In this study, we show that deletion of Ctcf in cells with active LysM in mice resulted in a mild decline in numbers of neutrophils, monocytes and macrophages in some peripheral tissues. Our observation that gene expression differences were more pronounced in macrophage cultures with lower Ctcf mRNA levels support the notion that a mild phenotype observed *in vivo* may be due to residual Ctcf protein. It is unclear what may cause possible retention of the Ctcf protein in myeloid cells. Expression of lacZ reporter demonstrated specific deletion of Ctcf in myeloid cells, but this does not exclude the possibility that in some cells incomplete deletions of *Ctcf* on both alleles may have occurred. The literature showing complete deletion of floxed transgenes using the LysM-Cre system (23, 46) together with our data showing reduction of Ctcf expression down to 25% of the wild type levels do not support this notion. We rather envisage that due to the fast turnover of myeloid cells in mice leading to a short time between the deletion event and analysis combined with a long half-life of the Ctcf protein in cells could underlie the observed differences in residual Ctcf protein levels.

Macrophages of *LysM-Cre Ctcff/f* mice demonstrated normal phagocytosis capacity and production of inflammatory cytokines IL-12 and IL-6, but decreased production of the cytokines TNF and IL-10, pointing to a confined change in functionality as a result of deletion of Ctcf. Our Ctcf ChIP-seq analysis provided evidence for strong Ctcf binding in the ~10 kb region encompassing *Tnf, Lta* (lymphotoxin-a) and *Ltb* genes, as well as in the loci encoding IL-6 and IL-12p40 (RWH, unpublished). Further experiments are required to investigate if Ctcf acts as direct regulator of the *Tnf-Lta-Ltb* locus or why Ctcf does not appear to regulate expression of the *Il6* or *Il12b* genes. Implications of these findings may be important in pathological conditions to influence production of cytokines without affecting other macrophage functions. Regulation of IL-10 or TNF produced by macrophages could improve immunotherapy of tumors by reducing unwanted IL-10 production and induction of immunosuppressive macrophages (47)-(48). Likewise, controlled induction of IL-10 or TNF in diabetes could improve wound healing or the regulation of the autoimmune response (49, 50).

Acknowledgments

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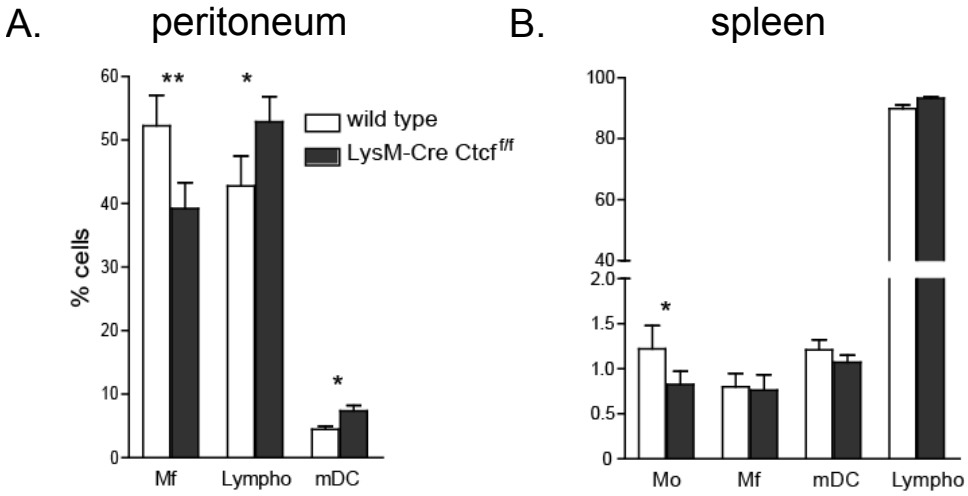
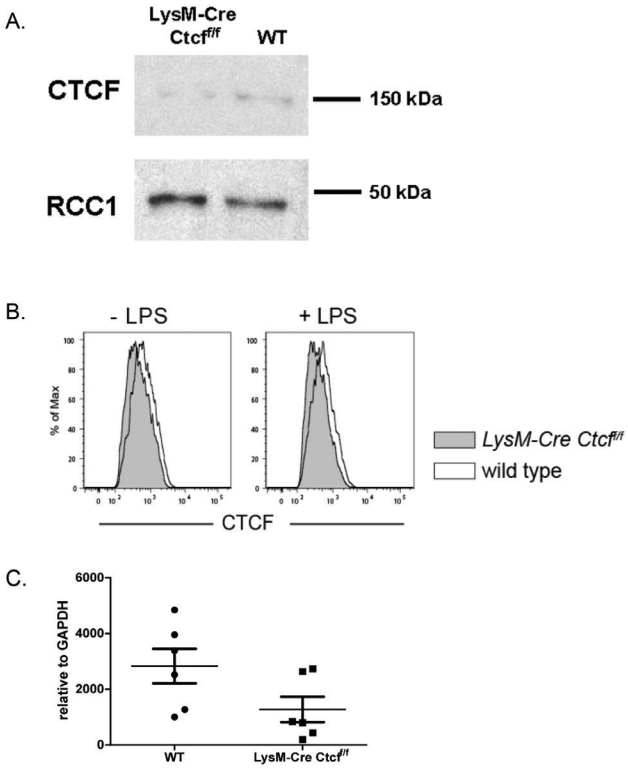
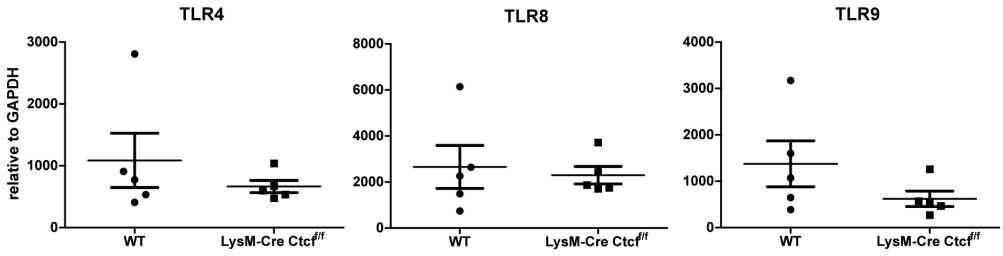
Supplementary Data



Supplementary Figure S1. Reduced frequency of myeloid cells in *LysM-Cre Ctcf$^{f/f}$* mice *in vivo*. Percentage of monocyte, macrophages, myeloid DC (mDC) and lymphocytes (% of the total leukocyte pool) in the peritoneum (A) and spleen (B) was determined using flow cytometry. Per organ, a combination of cell specific markers was evaluated to define cell types as: monocytes (SSC^{lo} CD11b^{hi} Ly6G^{-} Ly-6C^{+}), macrophages (CD11c^{low} CD11b^{hi} F4/80^{+}), mDC (B220^{-} CD11c^{hi}) and lymphocytes (CD11b^{-} CD11c^{-} and B220^{+}, CD4^{+} or CD8^{+}). The percentages of macrophages in the peritoneum and monocytes in the spleen are significantly reduced in *LysM-Cre Ctcf$^{f/f}$* mice when compared to wild type mice. White bars represent data from *LysM-Cre Ctcf$^{f/f}$* mice and black bars represent data from wild type mice. Statistical analysis: Wilcoxon T test, *p<0.05 and **p<0.01. Data show average frequencies ± SEM derived from 16 mice of each genotype.



Supplementary Figure S2. Expression of CTCF in cultured macrophages. (A) CTCF protein expression by cultured macrophages as determined by Western blot. Expression of chromatin-bound protein RCC1 was used as control for protein loading. Despite low expression of CTCF protein in wild type macrophages, reduced expression of CTCF in *LysM-Cre Ctcf$^{f/f}$* macrophages can be appreciated. (B) Intracellular staining of CTCF protein in cultured wild type and *LysM-Cre Ctcff/f* macrophages before and after stimulation with LPS. (C) CTCF mRNA levels from BM-derived macrophages from wild type (n=6) and *LysM-Cre Ctcf$^{f/f}$* (n=6) mice were quantified by real time PCR and expressed relatively to GAPDH. Bone marrow derived macrophages from *LysM-Cre Ctcf$^{f/f}$* mice have lower CTCF mRNA levels compared to that of wild type mice.



Supplementary Figure S3. The expression levels of TLR mRNA are similar in wild type and *LysM-Cre Ctcf$^{f/f}$* macrophages. TLR4, TLR8 and TLR9 mRNA expression in bone marrow derived macrophages from wild type macrophages (n=5) and *LysM-Cre Ctcf$^{f/f}$* macrophages (n=5) was quantified by real-time PCR. TLR mRNA expression was calculated relative to GAPDH mRNA.

Supplementary Table 1 Gene-specific primers used for quantitative RT-PCR analysis

Gene ID	NCBI ID		Primer sequence
GAPDH	NM_008084.2	FW	CGTCCCGTAGACAAAATGGT
		RV	TCTCCATGGTGGTGAAGACA
Ctcf		FW	GATCCTACCCTTCTCCAGATGAA
		RV	ACCTGTTCCTGTGACGGTAC
IL-10	NM_010548.2	FW	CACAGGGGAGAAATCGATGACA
		RV	ATTTGAATTCCCTGGGTGAGAAG
IL-19	NM_001009940.1	FW	AAGCCACCAATGCAACTAGGA
		RV	CCTAGAGACTTAAGGGCAGCAGAT
IL-20	NM_021380.1	FW	CAACCAAATTCTGAGTCACTTCATAGA
		RV	GAATGCCTAGTTCTCCCAAAGC
IL-24	NM_053095.2	FW	CAGCCCAGTAAGGACAATTCCA
		RV	CCAAAGCGACTTCTGTATCCAA

Abbreviation: Ctcf, CCCTC-binding factor.

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5

Inflammatory monocytes are the central instigators of early virus-induced liver inflammation

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6

Response to in vivo TLR7 ligation differs according to the kinetics of systemic and hepatic inflammation after LCMV infection

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7

General discussion and future perspectives

Summary

This study was performed to elucidate the immunological role of the liver in viral hepatitis. The immune functions of the liver are shaped by the intrahepatic cells present during steady state condition, as well as the recruited immune cells during liver inflammation (Figure 1). Liver resident Kupffer cells, by performing endocytosis, determine the functionality of the liver as a filtering organ. Additionally, Kupffer cells produce IL-10. This function, regulated by *Ctcf,* suggests the potential of Kupffer cells to perform immunoregulation. Monocytes patrol the liver in the steady state, but accumulate in the liver during viral hepatitis. They show functional versatility as demonstrated in the distinct polarization towards TNF-producing and endocytic cells in LCMV-infected and LPS-treated livers, respectively. During LCMV-induced hepatitis, fractions of both Kupffer cells and inflammatory monocytes alter their F4/80 expression, posing a challenge in immunological studies using flow cytometry. Furthermore, in this study we describe distinct clinical responses induced by TLR7 treatment at different phases of LCMV infection, which are associated with distinct states of immune activation.

Results from our study contribute to better understand the regulation of intrahepatic immune responses in the steady state condition and during viral hepatitis. Better insights in the functions of Kupffer cells and inflammatory monocytes will open up their potential to be targeted by HBV and HCV therapy. Furthermore, this study emphasizes the importance of characterizing the intrahepatic immune responses during chronic viral hepatitis to understand the mechanisms for the induction of adverse side-effects by TLR7 treatment. This information is valuable in order to prevent or predict the clinical outcome of TLR7-based treatment of HBV or HCV patients. Although this need to be validated in more detail, our findings suggest the significance of evaluating the TLR7 expression levels, either intrahepatic or systemic, in chronically infected patients prior to TLR7 treatment to minimalize the occurrence of adverse side-effects.

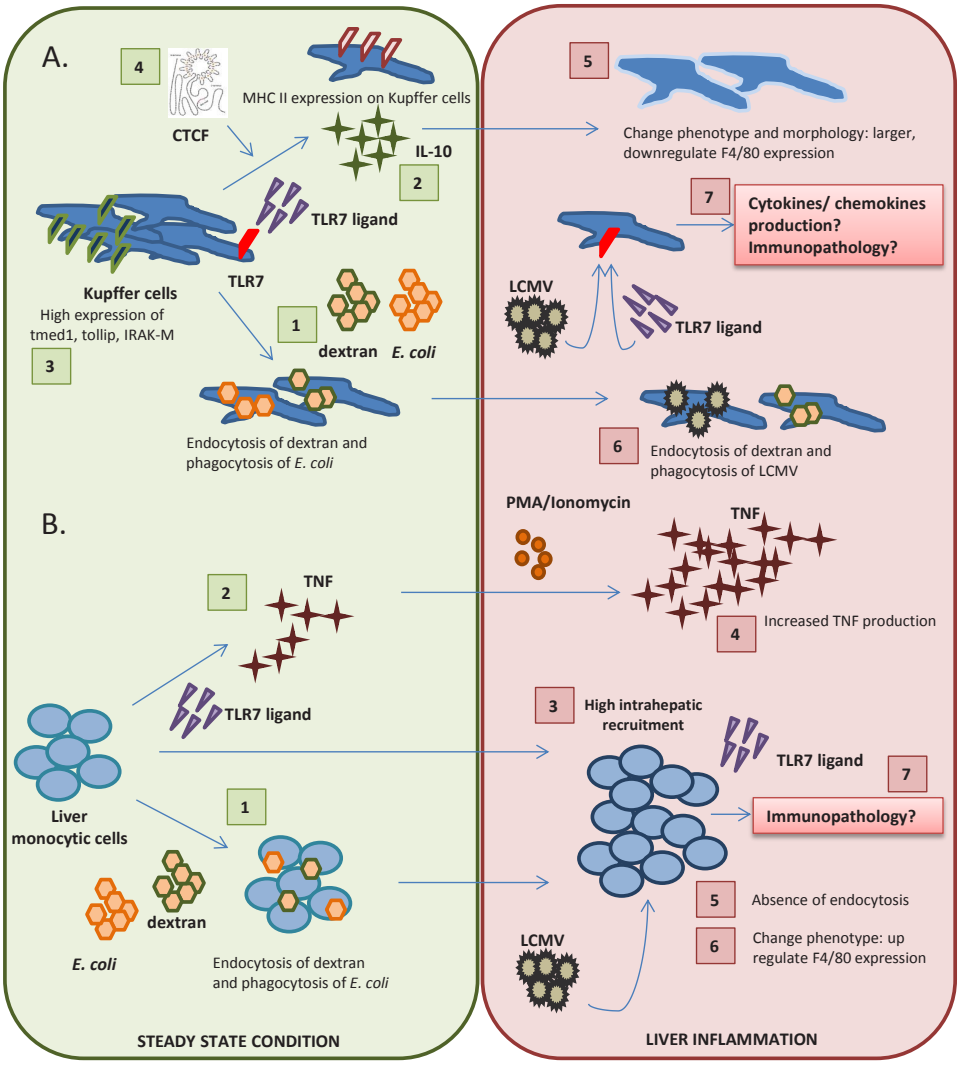
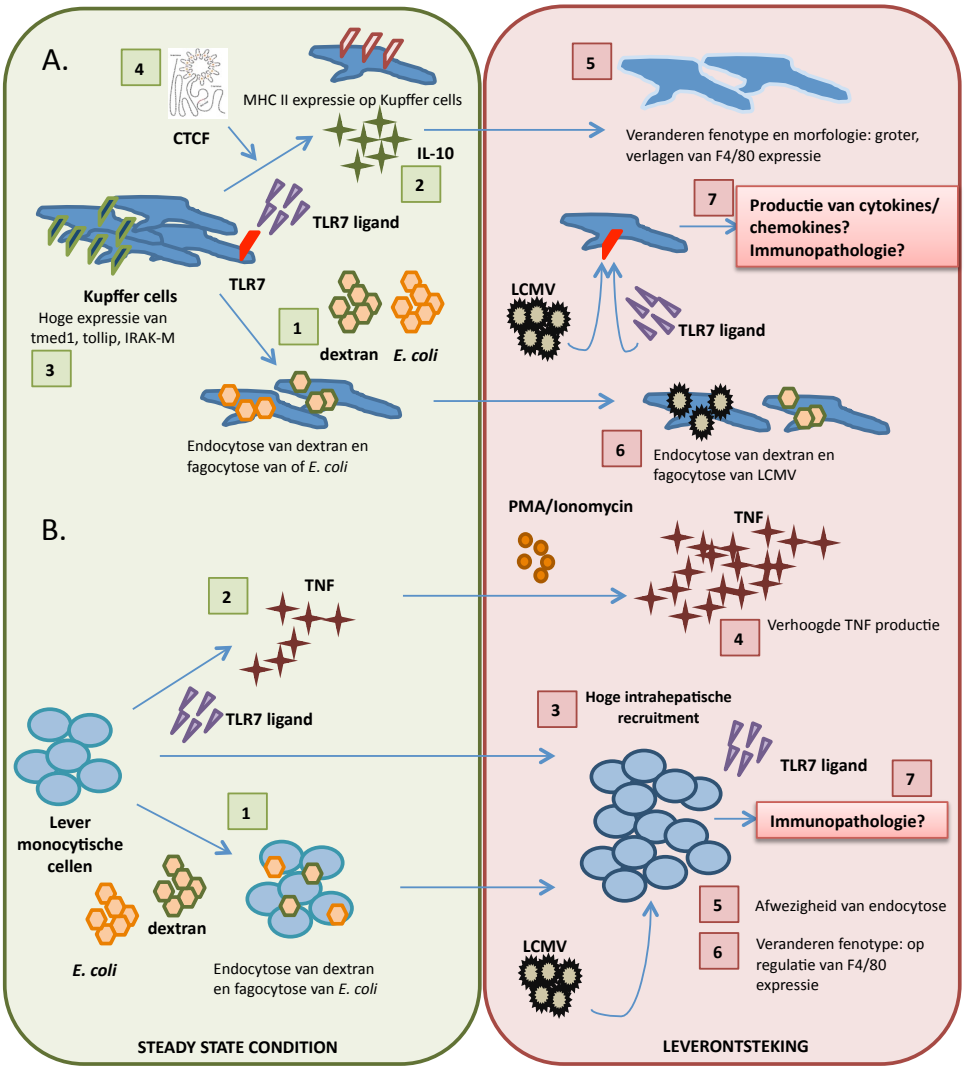


Figure 1. The immune functions of Kupffer cells and liver-derived monocytes. A) In the steady state condition, Kupffer cells are endocytic and phagocytic (1), produce IL-10 upon *in vitro* TLR7 ligation (2) but not IL-12p40 or TNF due to the high expression of tmed1, tollip and IRAK-M (3). IL-10 production and MHC II expression on Kupffer cells are regulated by *Ctcf* (4). During LCMV infection, Kupffer cells downregulate F4/80 expression and become larger (5), while retaining their endocytic and phagocytic abilities (6). It is currently not clear whether Kupffer cells produce inflammatory cytokines and chemokines during LCMV infection alone or in combination with TLR7 treatment (7). B) Liver monocytic cells are endocytic and phagocytic (1) and produce TNF upon *in vitro* TLR7 ligation (2). They are recruited to the liver during LCMV infection, thus termed inflammatory monocytes (3), and increase their TNF-producing ability (4). LCMV infection results in diminution of endocytic ability of inflammatory monocytes (5) and upregulation of F4/80 expression on these cells (6). Due to their abundance in the liver during the early phase of LCMV infection, inflammatory monocytes may indirectly induce immunopathology due to TLR7 treatment in LCMV-infected mice (7).

Samenvatting

Deze studie werd uitgevoerd om de immunologische rol van de lever bij virale hepatitis te verduidelijken. De immunologische functies van de lever worden gevormd door de intra hepatische cellen die aanwezig zijn tijdens de steady state toestand, alsmede de immuun cellen die tijdens leverontsteking worden gerekruteerd (Figuur 1). Door het uitvoeren van endocytose, bepalen de aanwezige Kupffer cellen de functionaliteit van de lever als filter orgaan. Verder produceren Kupffer cellen IL-10. Dit kenmerk, gereguleerd door *Ctcf*, suggereert dat Kupffer cellen immuun regulerende potentie hebben. In steady state patrouilleren de monocyten in de lever, maar tijdens een virale ontsteking hopen ze zich op. Ze vertonen een functionele veelzijdigheid, aangetoond door hun verschillende polarisatie naar TNF-producerende en endocytotische cellen in respectievelijk LCMV geïnfecteerde en LPS-behandelde levers. Tijdens LCMV geïnduceerde leverontsteking, veranderen fracties van zowel Kupffer cellen als inflammatoire monocyten hun F4/80 expressie, wat een probleem vormt om met behulp van flowcytometrie immunologische studies uit te voeren. Verder beschrijven we in deze studie verschillende klinische responses welke geïnduceerd worden door TLR7 behandeling in verschillende fases van LCMV infectie, deze worden geassocieerd met verschillende stadia van immuun activatie.

Resultaten van onze studie dragen bij aan een beter inzicht in de regulatie van intra hepatische immuunreacties tijdens de steady state en virale hepatitis. Een beter inzicht in de functies van Kupffercellen en inflammatoire monocyten opent hun potentie als doelwit tijdens HBV en HCV-therapie. Bovendien benadrukt deze studie het belang om tijdens chronische leverontsteking de intra hepatische immuunreacties te karakteriseren zodat we een beter inzicht krijgen in mechanismen die leiden naar negatieve bijwerkingen door TLR7 behandeling. Deze informatie is waardevol om de klinische uitkomst te voorkomen of te voorspelen tijdens TLR7 gebaseerde behandeling van HBV of HCV patiënten. Hoewel dit in meer detail moet worden bevestigt, suggereren onze bevindingen het belang van het vaststellen van het niveau van TLR7 expressie, hetzij intra hepatisch of systemisch in chronisch geïnfecteerde patiënten voorafgaand aan TLR7 behandeling om negatieve bijwerkingen te minimaliseren.



Figuur 1. De immunologische functies van Kupffercellen en lever-afgeleide monocyten. A) In de steady state kunnen Kupffer cellen fagocyteren en endocyteren (1), ze produceren IL-10 na *in vitro* stimulatie van TLR7 (2) maar niet IL-12p40 of TNF vanwege de hoge expressie van tmed1, tollip en IRAK-M (3). De IL-10 productie en MHC-II expressie op Kupffer cellen wordt gereguleerd door *Ctcf* (4). Tijdens LCMV infectie verlagen Kupffer cellen hun F4/80 expressie en worden ze groter (5), met behoud van hun endocytische en fagocytische kenmerken (6). Het is nog onduidelijk of Kupffer cellen alleen of in combinatie met TLR7 behandeling inflammatoire cytokines en chemokines produceren tijdens LCMV infectie (7). B) Monocytische levercellen kunnen endocyteren en fagocyteren (1) en produceren TNF na *in vitro* stimulatie van TLR7 (2). Tijdens LCMV infectie worden zij gerekruteerd naar de lever, daardoor inflammatoire monocyten genoemd (3), en verhogen hun TNF-producerend vermogen (4). LCMV infectie resulteert in vermindering van het endocytische vermogen van inflammatoire monocyten (5) en in op regulatie van F4/80 expressie op deze cellen (6). Door hun grote aantallen in de lever tijdens de vroege fase van LCMV infectie kunnen inflammatoire monocyten mogelijk indirect immunopathologie induceren als gevolg van TLR7 behandeling in LCMV geïnfecteerde muizen (7).

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PhD Portfolio

Name PhD candidate	Dowty Movita
Department	Gastroenterology and Hepatology, Erasmus MC
PhD period	January 2009 – September 2014
Promotor	Prof. dr. H.L.A. Janssen
Copromotor	Dr. P.A. Boonstra

PhD training

General academic and research skills (Erasmus MC)	*Year*
Introduction to data analysis (ESP03)	2009
Principles of Research in Medicine (ESP01)	2009
Animal experimentation (Article 9)	2009
Safety of radioactivity (niveau 5B)	2010
The advanced course of molecular immunology	2010
English Biomedical Writing and Communication	2011

(Inter)national conferences	*Year*
Dutch Liver Retreat, Spier, The Netherlands	2010
Dutch Society of Immunology, Lunteren, The Netherlands	2010
Annual Meeting European Macrophage and Dendritic cells Society (EMDS), Edinburgh, UK	2011
Dutch Society of Gastroenterology and Hepatology, Veldhoven, The Netherlands	2011
EASL Monothematic Conferences: Immune Mediated Liver Injury. The European Association for the Study of the Liver, Birmingham, UK	2012
Dutch Society of Immunology, Noordwijkerhout, The Netherlands	2012
Dutch Society of Gastroenterology and Hepatology, Veldhoven, The Netherlands	2013

Scientific Awards and Grants	*Year*
Trustfonds travel grant	2011
NVVI travel grant	2011
Trustfonds travel grant	2012

Invited speaker	*Year*
Dutch Liver Retreat, Spier, The Netherlands	2010
Dutch Society of Gastroenterology and Hepatology, Veldhoven, The Netherlands	2013

Curriculum Vitae

Dowty Movita was born on January 18, 1978 in Bandung, West Java province, Indonesia to parents B.S. Trisno and Melania. She obtained her Bachelor and professional degree in Pharmaceutical Sciences from Institut Teknologi Bandung, the oldest technology-oriented university in Indonesia. For the bachelor thesis, she studied the effect of ethanol and propylene glycol as penetrant enhancers on the *in vitro* diffusion of ketoprofen from the carbopol 940 gel. During her study, she developed a great interest in research. Soon after graduation she started her research-oriented career at PT Medion and PT Bio Farma, a veterinary and human vaccine companies, respectively, at the Research and Development Department. Working in these vaccine companies particularly encourages her interest in molecular and cellular biotechnology.

In 2005, Dowty was awarded a STUNED (Study in the Netherlands) scholarship and joined a master programme of biotechnology at Wageningen University, specializing in molecular and cellular biotechnology. She very much valued her time and experience during this study since she was able to join the research group of Prof. dr. Monique van Oers at the Virology Laboratory Wageningen University, to perform a master research study in line with her interest, a study on the biological activities of recombinant carp cytokines expressed in insect cells. Upon completion of the master study, she continued her collaboration with Prof. dr. Monique van Oers working on the baculovirus expression system to improve the expression of recombinant influenza hemagglutinin.

In 2009, Dowty started her PhD training at the Immunology of Viral Hepatitis group, focusing on the intrahepatic immune responses, delivered by particularly Kupffer cells and liver-derived monocytes from healthy and viral-infected livers. This study is promoted by Prof. dr. Harry Janssen and supervised by Dr. Andre Boonstra. She found this as an excellent opportunity to get trained on the immunological research using animal models which is indispensable in therapeutical research. This study yielded in several key findings which were published in international journals.

At this moment, Dowty continues her research activities in vaccine development at Crucell, the Netherlands. She wishes to gain more expertise in vaccine development before returning to Indonesia, to share her experience and knowledge and hopefully to support nourishing research in Indonesia.

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