Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by

M.Sc. Volker Klöss
born in: Agnetheln
Oral-examination: July 5, 2017

Cooperation of innate immune cells during Hepatitis C virus infection

Referees: Prof. Dr. Alexander Dalpke
PD Dr. Volker Lohmann

List of Abbreviations

ANXA2	Annexin A2
APC	Antigen presenting cell
ATP	Adenosine triphosphate
Baf	Bafilomycin
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophages
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
c-NS2	HCV proteins from core to NS2
Cq	Chloroquine
CT	Threshold cycle number
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DcR	Decoy receptor
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimehtylsulfoxide
DNA	Deoxyribonucleic acid
DR	Death receptor
ds	Double stranded
DV	Dengue virus
E	Envelope
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
ER	Endoplasmatic reticulum
ESCRT	endosomal sorting complex required for transport
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death receptor
FasL	Fas ligand
FCS	Fetal calf serum
Fig.	Figure
FISH	fluorescence in situ hybridization
FL2	Firefly Luciferase 2
GM-CSF	Granulocyte macrophage colony-stimulating factor

h	Hours
HAV	Hepatitis A virus
HCV	Hepatitis C virus
IFN	Interferon
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
IRES	Internal ribosome entry site
IRF	Interferon regulatory factors
ISG	Interferon stimulated genes
JAK	Janus kinase
JFH	Japanese fulminant hepatitis
KO	Knockout
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
Luc	Luciferase
MACS	Magnetic activated cell sorting
MAVS	Mitochondrial antiviral signaling protein
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony-stimulating factor
MDA5	Melanoma differentiation-associated protein 5
min	Minutes
MIP	Macrophage inflammatory protein
MOI	Mulitplicity of infection
MVB	Multi vesicular bodies
NCR	Non-translated region
NK cell	Natural killer cell
NS	Non-structural
ns	not significant
NTR	Non-translated region
PAMP	Pathogen associated molecular pattern
PARP	Poly(ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	plasmacytoid dendritic cell
PRR	Pattern recognition receptor
qPCR	Quantitative real time PCR
rE	Relative expression

RIG-I	retinoic acid-inducible gene-I
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SDS	Sodiumdodecyl sulfate
SGR	Subgenomic replicon
si	Small interfering
SMPDL3B	Sphingomyelin phosphodiesterase acid like 3B
SR-BI	Sscavenger receptor-BI
ss	Single stranded
STAT	Signal transducer and activator of transcription
SVR	Sustained virologic response
TLR	Toll-like receptor
TNF	Tumor necrosis factors
TRAIL	Ttumor necrosis factor-related apoptosis inducing ligand
TRIF	TIR-domain-containing adapter-inducing interferon-β
v/v	volume per volume
VLDL	Very low density lipoprotein
w/v	weight per volume

Table of contents

1 Summary

1.1 Abstract

Induction of an innate immune response requires recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). However, pathogens like the Hepatitis C virus (HCV) have evolved mechanisms to evade innate immune responses in infected host cells. HCV specifically infects hepatocytes, thus specialized immune cells are not affected by the immune modulatory mechanisms exerted by HCV. Indeed, it was observed that plasmacytoid dendritic cells (pDCs) recognize HCV infected cells and respond with IFNα secretion. In this study the response of innate immune cells against HCV was further analyzed in a co-culture of HCV subgenomic replicon (SGR) cells and peripheral blood mononuclear cells (PBMCs).

It was confirmed that IFNα is produced by pDCs in this co-culture and it was shown that the secreted IFNα inhibits viral replication. Subsequently, it was demonstrated that pDC activation is triggered by viral RNA and that recognition of viral RNA in pDCs is dependent on TLR7. The transfer of viral RNA from infected cells into pDCs required cell-cell contacts. Following the characterization of the pDC response, a Luminex assay was performed to detect further cytokines and chemokines secreted by PBMCs in response to HCV SGR cells. As secretion of IFNγ was detected, NK cells were assumed to be activated. NK cell depletion from PBMCs resulted in loss of IFNγ, proving that NK cells are the source of IFNγ. Moreover, it was observed that also monocytes play a role in the anti-viral response. Flow cytometry and ImageStream analysis revealed an uptake of particles from HCV SGR cells by monocytes. The particles were found to be apoptotic vesicles, as PBMCs induced apoptosis in HCV SGR cells via tumor necrosis factor-related apoptosis inducing ligand (TRAIL) expression on NK cells. However, interactions between pDCs, NK cells and monocytes were required for efficient clearance of HCV SGR cells, as purified immune cells alone did not kill HCV SGR cells. Although expression of TRAIL receptors was comparable in HCV SGR cells and Huh-7 control cells, HCV activated PBMCs specifically targeted HCV SGR cells without killing Huh-7 control cells. The specific kill of HCV SGR cells was finally explained by the observation that HCV SGR cells are sensitive towards TRAIL induced apoptosis.

In summary it was shown that next to pDCs also NK cells and monocytes contribute to the response against HCV. Most importantly, the results underline the necessity of the interplay and mutual activation of different innate immune cells to initiate an efficient, rapid and specific response against HCV infected cells.

1.2 Zusammenfassung

Eine Antwort des angeborenen Immunsystems wird durch die Erkennung von Pathogen-assoziierten molekularen Mustern durch Mustererkennungsrezeptoren ausgelöst. Pathogene wie das Hepatitis C Virus (HCV) verfügen jedoch über Mechanismen, die die angeborene Immunantwort in infizierten Zellen zu unterdrücken. HCV infiziert spezifisch Leberzellen, wodurch spezialisierte Immunzellen nicht von den immun-modulatorischen Mechanismen des Virus betroffen sind. Tatsächlich wurde beobachtet, dass plasmazytoide Dendritische Zellen (pDC) HCV infizierte Zellen erkennen und IFNα sekretieren. In dieser Arbeit wurde die Antwort des angeborenen Immunsytems gegen das HCV näher untersucht, indem HCV subgenomische Replikonzellen (HCV SGR Zellen) mit Immunzellen aus dem Blut (PBMCs) ko-kultiviert wurden.

Es wurde bestätigt, dass in der Ko-Kultur von PBMCs mit HCV SGR Zellen IFNα von pDCs produziert wird und dass IFNα die virale Replikation inhibiert. Anschließend wurde gezeigt, dass die pDC Aktivierung durch virale RNA ausgelöst wird und dass diese durch TLR7 in pDCs erkannt wird. Der Transfer viraler RNA von infizierten Hepatozyten in pDCs erforderte direkten Zell-Zell Kontakt. Nachdem die pDC Antwort charakterisiert wurde, wurde eine Luminex Messung durchgeführt, um weitere Zytokine und Chemokine, die in dieser Ko-Kultur von PBMCs sekretiert werden, zu entschlüsseln. Die Detektion von IFNγ lieferte einen Hinweis, dass NK Zellen an der Immunantwort gegen HCV beteiligt sind. Dies wurde bestätigt durch Depletion von NK Zellen aus PBMCs: unter diesen Bedingungen konnte kein IFNγ mehr gemessen werden. Darüber hinaus wurde entdeckt, dass auch Monozyten in der anti-viralen Immunantwort eine Rolle spielen. Durchflusszytometrie und ImageStream Analysen zeigten eine Aufnahme von Partikeln von HCV SGR Zellen durch Monozyten. Diese Partikel wurden als apoptotische Vesikel identifiziert, da PBMCs durch Expression von TRAIL auf NK Zellen Apoptose in HCV SGR Zellen auslösten. Für eine effiziente Eliminierung von HCV SGR Zellen waren jedoch Interaktionen zwischen pDCs, NK Zellen und Monozyten nötig, da isolierte Immunzellen HCV SGR Zellen nicht abtöten konnten. Obwohl die Expression der TRAIL Rezeptoren in HCV SGR Zellen und Huh-7 Kontrollzellen vergleichbar war, töteten durch HCV aktivierte PBMCs ausschließlich HCV SGR Zellen, nicht aber Huh-7 Kontrollzellen. Diese Spezifität konnte letztlich dadurch erklärt werden, dass HCV SGR Zellen per se sensibler gegenüber TRAIL induzierter Apoptose waren.

Zusammenfassend wurde gezeigt, dass neben pDCs auch NK Zellen und Monozyten zur Immunantwort gegen HCV beitragen. Die wichtigsten Erkenntnisse sind die Beobachtungen, dass nur durch ein Zusammenspiel und gegenseitige Aktivierung von verschiedenen Zellen des angeborenen Immunsystems eine effiziente, schnelle und spezifische Immunantwort gegen HCV infizierte Zellen ausgelöst wird.

2 Introduction

In our everyday life we are constantly challenged by various pathogens such as bacteria, viruses or fungi. It is the role of the immune system to recognize and combat those pathogens. In order to detect pathogens the immune system recognizes foreign structures which are different from the host (self versus non-self discrimination). The immune system consists of the innate and the adaptive immune system. The innate immune system is the first line of defense. It is specialized in detecting a broad range of so called pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs)[1]. In consequence, the immune response elicited by the innate immune system is not specific for a certain pathogen, but a more general response. However, specialized antigen-presenting cells (APCs) of the innate immune system are able to present foreign structures on their surface. Thereby, cells of the adaptive immune system are activated and initiate a specific response against the invading pathogen[2].

Infection immunology is the area of research that focuses on the interplay between pathogens and the immune system. For example, upon infection with the Hepatitis C virus (HCV) several foreign structures like viral nucleic acid and viral proteins are present in the host. These structures could be recognized by PRRs which would result in an immune response. In this work the ability of the innate immune system to detect HCV was investigated. Moreover, it was analyzed how different innate immune cells respond to the virus and how they interact in the anti-viral response.

2.1 The Hepatitis C virus

The Hepatitis C virus (HCV) is a member of the genus Hepacivirus, which belongs to the family of the Flaviviridae[3]. Infection with HCV implies a high risk for an establishment of a persistent infection. About 170 million people worldwide suffer from chronic HCV infection and are at high risk to develop liver cirrhosis and hepatocellular carcinoma[4]. As a consequence of these diseases caused by chronic HCV infection, approximately 350000 people die annually[5].

HCV is an enveloped virus with an average diameter of 55 nm[6]. The enveloped capsid includes the genome of HCV, a positive single stranded (ss) RNA with a length of around 9.6 kb. This RNA is flanked by 5’ and 3’ non-translated regions and contains an internal

ribosome entry site (IRES)[7]. Due to the IRES the viral RNA is targeted to ribosomes, which allows a cap-independent translation. From the genome a single polyprotein consisting of approximately 3000 amino acids is translated. This polyprotein is cleaved into 10 different proteins by viral and cellular proteases co- and post-translationally[7,8]: core, envelope protein 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B. While the virus particle consists of the structural proteins core, E1 and E2, the non-structural proteins are involved in replication and assembly and function as viral proteases[9,10]. The genome organization and polyprotein processing together with the function of individual proteins is shown in detail in Fig. 2-1.

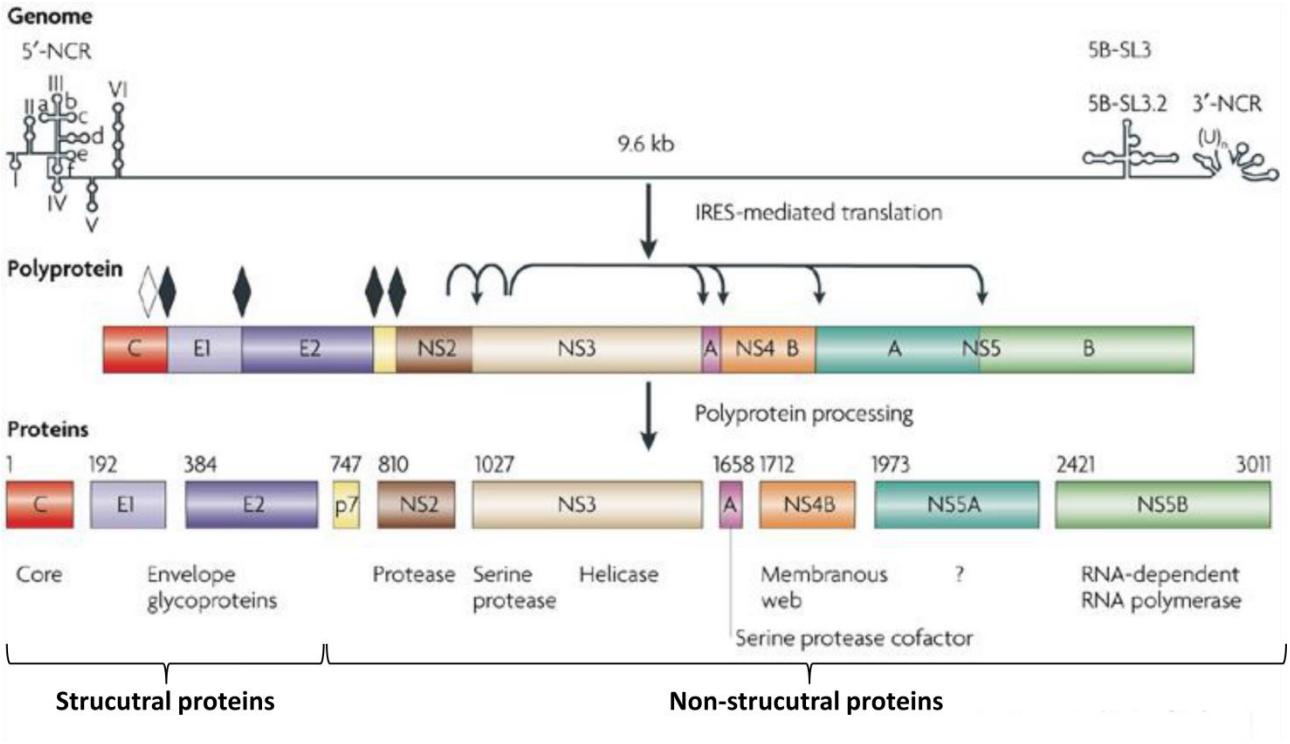

Fig. 2-1 The HCV genome and polyprotein organization
The HCV genome is schematically depicted with the 5' and 3' non-translated regions (NCR). Domains II-IV of the 5' NCR form an internal ribosome entry site (IRES). The IRES in the 5' NCR mediates translation of the HCV polyprotein precursor. The polyprotein is then processed co- and post-translationally into 10 proteins. Solid diamonds indicate cleavage sites of the HCV polyprotein precursor by an ER signal peptidase. The open diamond indicates further C-terminal processing of the core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2–3 and NS3–4A proteases. Below the single proteins are shown with their function and the general classification in structural and non-structural proteins is indicated. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, Moradpour et al.[7], copyright 2007.

2.1.1 HCV genotypes and treatment

Due to its high genomic diversity HCV is classified into 7 genotypes (1-7). These genotypes differ in their nucleotide sequence by 30–35%. Within the genotypes further subtypes are described which show differences in their sequence by 20-25%[11]. The genetic diversity of HCV is a major problem for the development of a protective vaccine against HCV infection. In the past, HCV infected patients (independent of the genotype) were treated with a combination of polyethylene glycol-conjugated Interferon-α (IFNα) with the nucleoside analogue ribavirin[12]. This therapy led to sustained virologic response (SVR, defined by the absence of HCV RNA in serum 24 weeks after treatment had been stopped) rates of 70-80% for genotype 2 or 3, but lower rates for other genotypes[13]. Later, therapy for genotype 1 had been adjusted to a triple therapy. In addition to IFNα, inhibitors of viral proteases (e.g. Boceprevir and Telaprevir) were administered, which significantly increased the rates of SVR[14]. Drawbacks of this therapy were severe side effects. In the next stage, many new drugs against HCV were in clinical trials. In particular, IFNα free treatment was investigated whereby side effects could be limited[15]. Nowadays patients with HCV infection can be cured by a combination of the NS5B inhibitor Sofosbuvir and the NS5A inhibitors Daclatasvir and Ledipasvir[16]. However, medication to prevent infections (e.g. vaccination) is not available.

2.1.2 HCV life cycle

HCV attaches to the host cell (hepatocytes) by binding to various receptors on the host cell surface. These are scavenger receptor-BI (SR-BI)[17], the tetraspanin co-receptor CD81[18] and the tight junction proteins Claudin[19] and Occludin[20]. Via clathrin-mediated endocytosis the virus then is internalized by host cells[21]. Inside the cell, viral RNA is released to the cytoplasm in a pH-dependent manner[22] and as described before can be translated directly due to its IRES. After expression of viral proteins, non-structural proteins rearrange ER membranes in the host cell. Thereby, a so called membranous web is formed, where viral replication is supposed to take place [23]. Initially, from the positive ssRNA genome a complementary negative strand is synthesized. Thus, double-stranded (ds) RNA occurs as a replication intermediate [24]. Likewise, also negative ssRNA is considered as a replication intermediate, as it serves as a template for synthesis of positive ssRNA. In turn, the progeny positive ssRNA is then used for further cycles of replication and translation or is

packaged into newly assembled virus particles. Viral particles exit the cell by highjacking the very low density lipoprotein (VLDL) pathway.

2.1.3 Fate of viral replication intermediates

As described before during HCV replication negative ssRNA and dsRNA occur as replication intermediates and both are potential activators of innate immunity (see 2.3.2). Interestingly, it has been observed that the ratio of positive and negative ss viral RNA is kept at a constant level[25]. Since viral positive ssRNA has a reported half-life between 11-14 h[26] also replication intermediates must be turned over to retain the constant ratio. However, it is currently unknown how turnover of these replication intermediates takes place. Unpublished data of AG Lohmann (Molecular Virology, Heidelberg) provide evidence that besides positive ssRNA[27] also negative ss viral RNA is secreted by HCV SGR cells. Thus, secretion seems to be a mechanism contributing to turnover of viral replication intermediates. Moreover, secretion of replication intermediates might contribute to immune evasion in the infected cell. In turn, release of viral RNA could also lead to activation of professional immune cells (see 2.3.2).

2.2 The Replicon System

For detailed studies of the intracellular stage of viruses the establishment of a permissive cell-culture system is a prerequisite. A milestone in the development of HCV cell culture systems was the development of subgenomic replicons (SGR)[28]. SGR are lacking the structural proteins. Hence, no virus particles can be formed. Instead they carry a specific resistance gene, allowing selection of the cells in which the SGR replicates. Moreover, SGRs contain an IRES of the encephalomyocarditis virus. This IRES allows translation of HCV proteins, while the HCV IRES directs translation of the resistance gene. The schematic setup of the SGR is shown in Fig. 2.

Most importantly, SGR are able to replicate autonomously after transfection into Huh-7 cells. Huh-7 is a human hepatoma cell line[29]. Interestingly, upon infection of Huh-7 cells with HCV no interferons are produced[30] (see below for immune escape mechanisms of HCV). Efficiency of replication for these SGR derived from genotype 1b (Con1 isolate) was increased by cell culture adapted mutations[31]. Later, a SGR from genotype 2a background

(JFH isolate) was found that replicated efficiently without the need of cell culture adaptive mutations[32]. Moreover, by introduction of a Luciferase gene into SGR, a tool was developed to easily monitor replication of the SGR by luciferase assays[33].

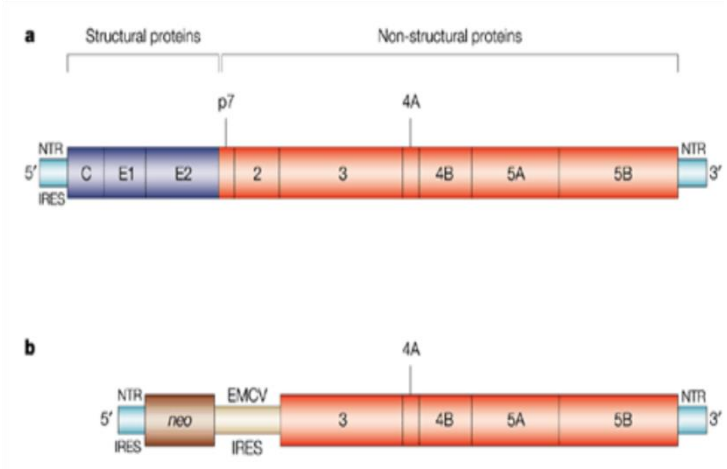

Fig. 2-2 Schematic organization of HCV subgenomic replicons
In (a) the HCV genome consisting of 5' and 3' non-translated regions (NTR), an internal ribosome entry site (IRES), structural proteins (blue) and non-structural proteins (red) is depicted. In (b) a subgenomic replicon is shown, in which the structural proteins were replaced by a neomycin phosphotransferase gene (neo) and an IRES of another virus (encephalomyocarditis virus; EMCV). This IRES directs the expression of the HCV non-structural proteins, whereas the selectable marker neo is translated under the control of the HCV IRES that is present in the 5' non-translated region (NTR). Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery, Bartenschlager et al.[34], copyright 2002.

2.3 Innate Immunity and HCV

2.3.1 The role of innate immunity

The innate immune system is the first line of defense against invading pathogens. It exerts a rapid, but rather unspecific response upon detection of a pathogen. Players of the innate immune system comprise physical factors (e.g. barrier of epithelial cell layers or mucus), the complement system as the humoral part and different cells such as granulocytes, natural killer cells (NK cells), monocytes and macrophages or dendritic cells (DCs). One function of innate immunity is to directly combat pathogens by different effector mechanisms (e.g. phagocytosis or cytokine production). Another important role is the activation of the highly specific adaptive immune response. Antigen-presenting cells (macrophages, DCs) present microbial components on their surface, where they are recognized by T lymphocytes. Especially DCs play an important role in this process, since naïve T-cells can on only be primed by DCs.

2.3.2 Pattern recognition receptors of viral RNA

In general, a prerequisite for initiation of an innate immune response is the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs)[1]. PAMPs are evolutionary conserved molecules, which are essential for microbial fitness. PRRs specifically recognize these conserved structures, which are absent in the host. Thereby, PRR distinguish between self and non-self. Next to PAMPs, also endogenous molecules, so called danger-associated molecular patterns (DAMPs) are recognized by PRRs[35]. For example, ATP under physiological conditions is only found inside of cells. Occurrence of extracellular ATP mediates a danger signal and ATP acts as a DAMP to activate immune cells[36].

Among the PRRs, certain Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) are detectors of viral RNA. TLRs that recognize nucleic acids are present in endosomes in order to avoid self-recognition. Essential for trafficking of endosomal TLRs from the ER to endosomes is the chaperone Unc93b1[37]. RLRs are present in the cytosol. TLR3 recognizes double stranded RNA[38], TLR 7 and 8 single stranded RNA[39,40] and RIG-I double stranded RNA or single stranded RNA with a 5' triphosphate[41,42]. Further RLRs detecting double stranded RNA are MDA5 and LPG2. Indeed, TLR7 and TLR8 polymorphisms[43,44] have been shown to play a role in HCV progression and RIG-I was shown to detect HCV RNA[45].

Although these receptors have specific signaling pathways, all can result in the expression of Interferon Regulatory Factors (IRFs). IRFs in turn drive expression of type I interferons (reviewed in[46]). IFNα and IFNβ are the most important type I interferons in viral defense. Upon binding of type I interferons to their respective receptor, a JAK-STAT signaling cascade is initiated which leads to expression of many IFN-stimulated genes (ISG). The concerted action of ISGs sets up an anti-viral state in which multiple anti-viral mechanism are employed[47].

However, pathogens have evolved mechanisms of immune evasion. HCV inhibits an immune response of the infected cell by cleaving MAVS (synonym for Cardif, VISA or IPS-1), an important adaptor molecule in RLR signaling, by its NS3/4A protease[48]. Furthermore, the NS3/4A protease also cleaves TRIF, an adaptor molecule in the TLR3 signaling pathway[49]. With these mechanism of immune evasion the production of type I interferons is strongly inhibited in HCV infected hepatocytes. However, an induction of

ISGs has been observed in HCV infected livers[50,51]. In consequence, it was hypothesized that interferons are produced by cells that are not infected by HCV. As HCV predominantly infects hepatocytes, immune cells were considered to be responsible for secretion of interferons. The role of innate immune cells in interferon secretion in response to HCV will be described in the following.

2.3.3 Plasmacytoid DCs and HCV

A specialized innate immune cell that produces high amounts of IFNα and IFNβ upon stimulation is the plasmacytoid dendritic cell (pDC)[52]. pDCs are a specific subset of dendritic cells that circulate through the blood and can be recruited to tissues. They express TLR7 and TLR9[53,54]. While TLR9 recognizes CpG rich DNA, TLR7 detects single stranded RNA and therefore might sense HCV RNA. Indeed, it has been shown that pDCs are present in high amounts in HCV infected livers[55]. However, some reports indicated an impaired function of pDCs upon HCV infection[56,57]. It was observed that patients with chronic HCV infection had reduced numbers of pDCs in the blood, that pDCs were less efficient in activation of CD4 T-helper cells and that pDCs secreted less IFNα. Nevertheless, other groups could show that HCV triggers IFNα production by pDCs. At first Gondois-Rey et al. described a weak activation of pDCs by HCV in comparison with influenza and herpes virus[58]. Later Takahashi et al. provided further evidence that pDCs sense HCV infected cells in co-culture experiments and produce large amounts of IFNα via TLR7 signaling[59]. Interestingly, this group observed that pDCs are also stimulated when co-cultured with HCV SGR cells instead of virus infected cells, but HCV virions alone did not stimulate pDCs. In consequence, it was speculated that active viral replication and cell-cell contacts between pDCs and HCV SGR cells were required for IFNα secretion by pDCs. Thus, this study provided evidence that ISG up-regulation in HCV infected livers is driven by IFNα secreted by pDCs.

The observation that pDCs were stimulated in co-culture raised the question how viral RNA is accessing pDCs. It is known that HCV SGR cells secrete viral positive ssRNA protected by membranes, although due to the lack of structural proteins no virus particles can be formed[27]. Furthermore, the finding that plasma from HCV patients contains exosomes associated with viral RNA[60] hinted towards a possible role of exosomal transport of viral RNA from infected cells to pDCs. Exosomes are 40-100 nm small

vesicles which are formed by an endocytosis event in late endosomes, which are then called multi vesicular bodies (MVB). Fusion of these MVB with the plasma membrane releases exosomes from the cell and many cell types are known to secrete exosomes (a detailed review of exosomes can be found in[61]). Since their discovery many roles of exosomes have been described. The most important role in this context is the transport of RNA between different cells[62]. It was shown that within the above described co-culture experiments a “short range transfer” of exosomes released from SGR cells triggered a response of pDCs via TLR7 signaling[63]. It was further shown that this transfer was dependent on the endosomal sorting complex required for transport (ESCRT) machinery, which was described to be involved in exosome formation[64–66]. Moreover, it was observed that expression of Annexin A2 (ANXA2) in hepatocytes was essential for pDC activation. ANXA2 was described to bind to RNAs[67,68] and to be required for vesicle trafficking and generation of MVBs[69–71]. It has also been linked to production of HCV virions[72] and to participate in the formation of HCV replication complexes[73]. In summary, the study by Dreux et al. described a route of transfer of viral RNA from infected cells into pDCs via “short range transfer” of exosomes [63].

2.3.4 Monocytes and HCV

Similar to pDCs, monocytes are part of the cellular innate immune system. They circulate through the blood and can be recruited to tissues upon infection. Within the population of peripheral blood mononuclear cells (PBMCs), monocytes can be detected by the expression of their surface marker CD14[74]. They are further subdivided into 3 populations based on the expression levels of CD16[75] and each population accounts for different functions (reviewed in[76]). Classic effector mechanisms of monocytes are the phagocytosis of bacteria and the secretion of pro-inflammatory cytokines[77]. Upon extravasation from the blood into tissues monocytes differentiate into macrophages[78]. Of note, tissue specific macrophages can also be generated during embryonic development (reviewed in[79]). Macrophages in addition act as antigen-presenting cells, thereby bridging innate and adaptive immunity.

Monocytes in theory could also be able to detect HCV RNA as monocytes express TLR8[80], which as described above recognizes ssRNA. However, thus far no evidence exists that monocytes respond to HCV RNA. In general, not much is known about the contribution of

monocytes in the anti-viral response against HCV. Over a long time research on the role of monocytes in HCV infection focused on the question if monocytes and macrophages can be infected by HCV, which is still discussed controversially (reviewed in[81]). Later, one study then showed that monocytes are able to sense HCV infected cells and to induce a response[82]. This study demonstrated that monocytes show inflammasome activation upon sensing HCV infected cells, but the underlying mechanism was not investigated. Moreover, it was shown that monocytes secreted TNFα and that inflammasome activation led to secretion of IL-18. These functions were impaired in monocytes from HCV infected patients. While TNFα had a direct effect on viral replication, IL-18 activated NK cells. IFNγ secretion by NK cells then in turn further boosted the anti-viral response. Thus, this study provided evidence that monocytes play a role in the immune response against HCV. Second, the results hinted towards an interplay between innate immune cells in the anti-viral response.

2.3.5 NK cell effector mechanisms

Despite being classified as lymphocytes, NK cells functionally belong to the cells of the innate immune system since they do not express antigen specific receptors. As their name “natural killer cells” already implies, they are specialized in killing virus infected cells[83,84] and tumor cells [85,86]. Tumor cells lacking MHC class I expression can be targeted by NK cells[87], as explained by the missing self hypothesis. In contrast to the above described active recognition of foreign structures by PRRs, NK cells can also be activated by the absence of “self” structures. Nowadays it is understood, that NK cell activation depends on recognition of various activating and inhibitory ligands (detailed review in[88]). NK cells can target cells by three different effector mechanisms. First, NK cells are able to secrete IFNγ[89]. Thereby, ISGs are up-regulated in target cells and viral infection is hampered[47]. Second, NK cells can kill target cells by secretion of cytotoxic granules[90]. These granules are similar to the granules released by cytotoxic T-killer cells and contain perforin and granzyme (reviewed in[91]). Third, by up-regulation of apoptosis inducing ligands like Fas ligand (FasL) and tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) NK cells can drive apoptosis of target cells. TRAIL is a member of the TNF family[92] and can bind to different receptors. These receptors can be classified in pro- or anti-apoptotic receptors. While binding of TRAIL to DR4[93] or DR5[94] induces apoptosis, binding to DcR1[95] or DcR2[96] protects from apoptosis, as these receptors lack intracellular signaling domains. In

contrast, DR4 and DR5 have an intracellular death domain. Upon TRAIL binding, homotrimers of the receptors are formed[97] and clusters of death domains are built. The adaptor protein Fas-associated death receptor (FADD) is recruited to this cluster, and in turn recruits caspase-8 and caspase-10. This leads to successive caspase activation via caspase-8, caspase-10, caspase-6, caspase-7 and caspase-3. Detailed reviews about the apoptotic signaling including several other players are given in Wang et al.[98] and in Gonzalvez et al.[99]. Caspase-3 finally cleaves numerous cellular proteins to initiate the programmed cell death of apoptosis. Among others, poly(ADP-ribose) polymerase (PARP) is cleaved by caspase-3 which determines the final irreversible step of apoptosis[100].

2.3.6 NK cells and HCV

NK cells were also described to play a role in the anti-viral response against HCV. Over 10 years ago it was reported that NK cells activated by cytokines can kill HCV SGR cells[101]. Later it was shown that IFNγ is secreted by NK cells in an HCV context. However, IFNγ secretion by NK cells was shown to be dependent on pDCs[102]. It was observed that in the co-culture of HCV SGR cells with PBMCs IFNγ was secreted and it was proven by flow cytometry that NK cells were the source of IFNγ. Of note, no IFNγ was secreted by purified NK cells in response to HCV SGR cells. Further experiments demonstrated that IFNα secretion by pDCs was essential for NK cell activation and that monocyte-derived IL-15 was needed for maximal IFNγ production. Interestingly, although they observed a role of cytokines in NK cell activation it was also shown that NK cells required contacts to other cells. However, it was not clear, if contacts with HCV SGR cells, with other immune cells or with both were necessary for NK cell activation. In contrast, another study described a predominant role for monocytes in NK cell activation[82]. As described above (see 2.3.4) this study showed that monocyte derived IL-18 triggered IFNγ secretion by NK cells, while pDCs had no influence.

Moreover, it has been described that IFNα in HCV infection can result in TRAIL expression on NK cells[103,104]. In the first study it was observed that chronic HCV infection increased the percentage of TRAIL positive NK cells, especially for NK cells in the liver compared to NK cells in the blood. Therefore, it was hypothesized that IFNα secretion in the liver might account for TRAIL expression on NK cells. Indeed they could show that in vitro stimulation of NK cells from healthy donors and from HCV patients with IFNα led to TRAIL expression

on NK cells. Stegmann et al. in the second study stimulated PBMCs from healthy donors with IFNα and found by microarray analysis that TRAIL was strongly up-regulated in NK cells. Similar to the first study they also stimulated NK cells from HCV patients with IFNα in vitro and as well observed an up-regulation of TRAIL. Interestingly, NK cells from patients that responded to IFNα therapy also displayed stronger TRAIL expression after in vitro stimulation with IFNα. Thus, the authors concluded that TRAIL expression on NK cells can control the virus.

In summary, these findings suggested that next to pDCs and monocytes also NK cells contribute to the anti-viral response against HCV. Importantly, in all studies NK cell activation was reported to be dependent on other innate immune cells like pDCs and monocytes. Hence, it became clear that innate immune cells interact with each other in the course of HCV infection. While previous studies mainly focused on the influence of monocytes and pDCs on NK cells, it was hypothesized that additional interactions between innate immune cells could boost the anti-viral response. Thus, in this work the analysis of the interaction and mutual activation between innate immune cells was a central part.

2.4 Objectives of this study

The aim of this work was to analyze the activation of innate immune cells by viral RNA secreted from HCV SGR cells. First, a co-culture of HCV SGR cells with PBMCs should be established to reproduce the findings that IFNα is produced in this co-culture. In the following this activation process should be further investigated. In detail, it should be investigated if recognition of viral RNA in fact is the trigger for activation of innate immune cells. Moreover, this study was planned to provide further evidence how viral RNA is transported from HCV SGR cells into immune cells. Thus, the role of exosomal transport of viral RNA and via direct cell-cell contact should be examined.

In addition, it was planned to further analyze if also other innate immune cells next to pDCs participate in the response against HCV. Literature provided first indications that also monocytes and NK cells play a role in the anti-viral response against HCV. Hence, this work should give deeper insights about their contribution. In particular, also the interaction between different innate immune cells in the response against HCV should be

addressed. The focus here laid on the question if a possible interaction and mutual activation of innate immune cells leads to a stronger and more efficient response.

3 Materials & Methods

3.1 Materials

3.1.1 Devices & Instruments

Device	Type & Manufacturer
AutoMACS	Miltenyi Biotec, Bergisch-Gladbach
Balance	EW600-2M, Kern & Sohn GmbH, Balingen
Blotting chambers	Transfer chamber/ Biometra, Jena
Centrifuges	Multi 3 S-R, Heraeus Instruments; Hanau Fresco17, Thermo Scientific, Karlsruhe
Counting chamber	Neubauer 0.00025 mm^2/0.1 mm, Brand GmbH, Schwerin
Fine balance	MC1 Research RC 210 P, Wiegetechnik Knoll, Ketsch
Flow cytometer	BD FACSCanto™, BD Biosciences, San Diego, USA
Heating blocks	AccuBlock, Eppendorf, Hamburg
ImageStream	IS100, Amnis, Merck Millipore, Darmstadt
Incubator	BBD6226, Heraus Instruments, Hanau
Luminometer	Mithras LB 940, Berthold technologies, Freiburg
Mass Spectrometer	Q-Exactive HF MS, Thermo Fisher Scientific, Waltham, USA
Microscope	Leica DM-LS, Leica GmbH, Wetzlar
Microtiter plate photometer	SUNRISE Absorbance Reader, Tecan, Salzburg, Austria
Orbital shaker	Labortechnik Fröbel GmbH, Lindau
pH-Meter	Seven Easy, Mettler Toledo, Gießen
Power supply	Power Pac HC, BioRad, München
Q-Exactive HF MS	Thermo Scientific, Karlsruhe
qPCR cycler	StepOne Plus, Applied Biosystems, Darmstadt
SDS-PAGE system	perfectBlue™ Twi S, Peqlab, Erlangen
Spectrophotometer	NanoDrop®ND-1000, Peqlab, Erlangen
Sterile bench	Hera Safe KS 12, Heraeus Instruments, Hanau
SW28 rotor	Beckman-Coulter, Krefeld
Thermocycler	Primus advanced 96, Peqlab, Erlangen
Vortexer	MS 1, IKA®Works, Inc., Wilmington, USA

3.1.2 Consumables

Consumables	Manufacturer
24-well cell culture plate	Greiner Bio-One, Frickenhausen
48-well cell culture plate	Corning Incorporated, Corning, USA
96-well cell culture plate	Greiner Bio-One, Frickenhausen
Blotting membrane	Immobilon-P Transfer, Millipore Billerica, USA
Blotting paper	Whatman® GB003, Whatman GmbH, Dassel
cDNA tubes	PP-PCR-tubes, Greiner Bio-One GmbH, Frickenhausen
Cell culture plates	Greiner Bio-One, Frickenhausen
Cell scraper	Greiner Bio-One, Frickenhausen
ELISA plates	96 well, Greiner Bio-One GmbH, Frickenhausen
Eppendorf tubes	Eppendorf AG, Hamburg
FACS tubes	BD Falcon™ 5 ml, BD Biosciences, Heidelberg
Falcon tubes	Greiner Bio-One, Frickenhausen
Gauge needles	Microlance™ 3, 27G, BD Biosciences, Heidelberg
Optical Adhesive Cover	Absolute QPCR Seal, ABgene House, Epsom, UK
Petri dishes	Greiner Bio-One, Frickenhausen
Pipette tips	Hinged Rack Pipette tips, Corning, New York, USA
qPCR plates	MicroAmp, Fast 96-well reaction plate (0.1 ml), AppliedBiosystems, UK
Syringe	BD Discardit II 27G, 10ml, BD Biosciences, Heidelberg
Transwell inserts	1 µm pore size, 24 well, Corning Incorporated, Corning, USA

3.1.3 Chemicals & Reagents

Chemical / Reagent	Manufacturer
Acrylamide, stock solution (30%)	Roth GmbH, Karlsruhe
Ammonium persulphate (APS)	Sigma-Aldrich, Taufkirchen
Aqua ad injectabila	Braun, Melsungen
Blasticidin	Sigma-Aldrich, Taufkirchen
Bovine serum albumin (BSA)	Sigma-Aldrich, Taufkirchen
Brefeldin A	Sigma-Aldrich, Taufkirchen
Bromphenol blue	Sigma-Aldrich, Taufkirchen
Carboxyfluorescein succinimidyl ester (CFSE)	Sigma-Aldrich, Taufkirchen
Coomassie Brilliant Blue R-250	Thermo Scientific, Karlsruhe

CytoTell™ Red 650	AAT Bioquest, Sunnyvale, USA
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen
Dithiothreitol	Sigma-Aldrich, Taufkirchen
DMEM	Biochrom AG, Berlin
Enhanced chemiluminescence (ECL) substrate	PerkinElmer, Rodgau
Ethanol	Sigma-Aldrich, Taufkirchen
FACSClean™	BD Bioscience, Heidelberg
FACSFlow™	BD Bioscience, Heidelberg
Fetal calf serum (FCS)	GibcoTM, Invitrogen, Karlsruhe
FL2 in vitro transcript	G. Stöcklin, DKFZ Heidelberg
G418	Life Technologies, Darmstadt
Heparin Solution	StemCell Techonolgies, Köln
Hygromycin B	Life Technologies, Darmstadt
Isopropanol	Riedel-de Haën AG, Seelze
L-glutamine	Life Technologies, Darmstadt
Lipofectamine RNAiMAX	Life Technologies, Darmstadt
MACS buffer	Miltenyi Biotec, Bergisch-Gladbach
Methanol	Riedel-de Haën AG, Seelze
OptiMEM	Invitrogen, Karlsruhe
Pancoll	PAN-Biotech GmbH, Aidenbach
PBS	PAA Laboratories, Pasching, Austria
Penicillin/Streptomycin (100x)	Sigma-Aldrich, Taufkirchen
Penicillin/Streptomycin solution	PAA Laboratories, Pasching, Austria
Puromycin	Sigma-Aldrich, Taufkirchen
RPMI	Biochrom AG, Berlin
Saponin	Carl Roth GmbH, Karlsruhe
Sodiumchloride	AppliChem GmbH, Darmstadt
Sodiumdihydrogenphosphate	AppliChem GmbH, Darmstadt
Sodiumdodecylsulfate (SDS)	AppliChem GmbH, Darmstadt
Sodiumphosphate	Merck, Darmstadt
Sulfuric acid	VWR International S.A.S, Fontenay-sous-Bois, France
TEMED	Sigma-Aldrich, Taufkirchen
Trypan blue	Sigma-Aldrich, Taufkirchen
Trypsin/EDTA	PAA Laboratories, Pasching, Austria
Tween-20	Sigma-Aldrich, Taufkirchen
β-mercaptoethanol	Sigma-Aldrich, Taufkirchen

3.1.4 Kits

Chemical / Reagent	Manufacturer
BD OptEIA™ human IL-6 ELISA set	BD Biosciences, Heidelberg
BD OptEIA™ human IL-6 ELISA set	BD Biosciences, Heidelberg
BD OptEIATM murine IL-6 ELISA set	BD Biosciences, Heidelberg
CD14 Microbeads isolation kit	Miltenyi Biotec, Bergisch-Gladbach
CD304 Microbeads isolation kit	Miltenyi Biotec, Bergisch-Gladbach
CD56 Microbeads isolation kit	Miltenyi Biotec, Bergisch-Gladbach
Cytotoxicity Detection Kit Plus (LDH)	Roche Diagnostics, Mannheim
Fast SYBR green master mix	Applied Biosystems, Darmstadt
High capacity cDNA reverse transcription kit	AppliedBiosystems, Darmstadt
IFNα ELISA kit human	eBioscience, Frankfurt
IFNγ ELISA kit human	eBioscience, Frankfurt
NucleoSpin® RNA kit	Macherey-Nagel, Düren
PageRuler™Prestained Protein Ladder Plus	Fermentas, St. Leon-Rot
PeqGold Total RNA Kit	Peqlab Biotechnology GmBH, Erlangen
Pierce Cell Surface Protein Isolation Kit	Thermo Scientific, Karlsruhe

3.1.5 Buffers and solutions

Buffer	Reagent Concentrations
10x PBS	80 mM di-sodiumhydrogenphosphate; 20 mM sodium di-hydrogenphosphate; 1.4 M NaCl; pH 7.4
10x TBS	100 mM Tris-HCl, pH 8.0; 1.5 M NaCl
1x TBST	1xTBS, 0.05% (v/v) Tween-20
4x SDS PAGE sample buffer	40% (w/v) glycerol, 8% (w/v) SDS, 400 mM (v/v) β-mercaptoethanol, 200 mM Tris-HCl (pH 8.8), 0.4% (w/v) bromophenolblue
ELISA blocking buffer (IFNα)	1 x PBS, 0.5% (w/v) BSA, 0.05% (v/v) Tween-20
ELISA blocking buffer	1x PBS; 10% (v/v) FCS
ELISA coating buffer	0.1 M sodiumcarbonate, pH 9.5
ELISA washing buffer	1x PBS; 0.05% (v/v) Tween-20
FACS buffer	1x PBS, 2% (v/v) FCS
Luciferase assay buffer	25 mM glycyl-glycin (pH 7.8), 15 mM potassium phosphate buffer (pH 7.8); 15 mM $MgSO_4$; 4 mM EGTA, 1 mM DTT, 2 mM ATP and 0.07 mM luciferin

Luciferase lysis buffer	1% Triton X-100; 25 mM glycyl-glycin (pH7.8); 15 mM $MgSO_4$; 4 mM EGTA; 10% glycerol, 1 mM DTT
Pen/Strep(100x)	10.000 U/ml penicillin G; 10 mg/ml streptomycin; 0.9% (w/v) NaCl
Permeabilization buffer	1x FACS buffer; 0.1% saponin
SDS-PAGE Running buffer	25 mM TRIS-OH (pH 8.3), 192 mM Glycin, 10% (v/v) Methanol
Semidry blotting buffer	25 mM Tris-OH (pH 8.3), 192 mM Glycin, 10% (v/v) Methanol
Separating gel buffer	1.5 M Tris-HCl (pH 8.8), 0.4% (w/v) SDS
Stacking gel buffer	1 M Tris-HCl (pH 6.8), 0.8% (w/v) SDS
Trypan blue solution	2 mg/ml trypan blue in 1x PBS
Trypsin solution	0.05% trypsin; 0.02% EDTA in 1x PBS

3.1.6 Primers for qPCR

Target	Forward (5`to 3`)	Reverse (5`to 3`)
DcR1	TACCACGACCAGAGACACC	CAGTGGTGGCAGAGTAAGC
DcR2	TACCACGACCAGAGACACC	CACCCTGTTCTACACGTCCG
DR4	AGAGAGAAGTCCCTGCACCA	GTCACTCCAGGGCGTACAAT
DR5	AAGACCCTTGTGCTCGTTGT	AGGTGGACACAATCCCTCTG
FL2	ATCACAGAATCGTCGTATGC	CATACTGTTGAGCAATTCACG
HCV	CGGTTCTTTTTGTCAAGACC	GAGCAAGGTGAGATGACAG
IL-8	CAAGAGCCAGGAAGAAACCA	GCAAAACTGCACCTTCACAC
IP-10	GGTCTGAGTGGGACTCAAGG	GTGGCAATGATCTCAACACG
MCP-1	ATAGCAGCCACCTTCATTCC	CAGATCTCCTTGGCCACAAT
MIP-1α	AGTTCTCTGCATCACTTGCTG	CGGCTTCGCTTGGTTAGGAA
MIP-1β	CTGTGCTGATCCCAGTGAATC	TCAGTTCAGTTCCAGGTCATACA
Rab27a	GGAGAGGTTTCGTAGCTTAACG	CCACACAGCACTATATCTGGGT
β-actin	GGCTCCGATATCTCTGTCGT	ATGTTGCATTTCGTCACACC

3.1.7 Antibodies

Target	Application	Manufacturer	Ref.nr.
IFNα	Neutralization	PBL, Piscataway, USA	21112-1
IgG1	Neutralization	Acris, San Diego, USA	AM03095PU-N

CD81	Blocking	BD Biosciences, Heidelberg	555675
CD9	Blocking	BD Biosciences, Heidelberg	555370
IgG1	Blocking	BD Biosciences, Heidelberg	555746
TRAIL	Blocking	BD Biosciences, Heidelberg	550912
Cleaved PARP	WB	Cell Signaling, Frankfurt	#9541
β-actin	WB	Cell Signaling, Frankfurt	#4970
Anti-Rabbit IgG	WB	Cell Signaling, Frankfurt	#7074
CD14 APC	FC	BD Biosciences, Heidelberg	555399
CD14 PE	FC	BD Biosciences, Heidelberg	562691
CD25 PerCP Cy5.5	FC	Biolegend, San Diego, USA	356112
CD3 FITC	FC	BD Biosciences, Heidelberg	555332
CD3 FITC	FC	BD Biosciences, Heidelberg	555332
CD3 APC Cy7	FC	BD Biosciences, Heidelberg	557832
CD38 APC	FC	BD Biosciences, Heidelberg	555462
CD44 FITC	FC	BD Biosciences, Heidelberg	555478
CD56 APC	FC	Miltenyi Biotec, Bergisch-Gladbach	130-090-843
CD56 Pe Cy7	FC	eBioscience, Frankfurt	25-0567-42
CD69 APC	FC	BD Biosciences, Heidelberg	555533
CD80 PE	FC	BD Biosciences, Heidelberg	557227
CD86 PE	FC	BD Biosciences, Heidelberg	555665
IL-8 APC	FC	eBioscience, Frankfurt	17-8088
TRAIL PE	FC	BD Biosciences, Heidelberg	550516

3.1.8 Inhibitors & Stimuli

Inhibitor / Stimulus	Manufacturer
Bafilomycin	Calbiochem, Darmstadt
Chloroquine	Sigma-Aldrich, Taufkirchen
CpG1668	Invivogen, Toulouse, France
GW4869	Cayman Chemical, Ann Arbor, USA
IFNα	Peprotech, Hamburg
IFNγ	Peprotech, Hamburg
Lipopolysaccharide (LPS)	purified from S. minnesota, kind gift from U. Seydel, Borstel
R848	Invivogen, Toulouse, France
Spiroepoxide	Santa Cruz Biotechnology, Dallas, USA
TNFα	Peprotech, Hamburg
TRAIL	eBioscience, Frankfurt

Z-IETD-FMK	Enzo Life Sciences, Lausen, Switzerland
Z-VAD-FMK	Invivogen, Toulouse, France
Z-YVAD-FMK	Enzo Life Sciences, Lausen, Switzerland

3.1.9 siRNA

Target	Sequence (5`to 3`)
Rab27a	GCUGCCAAUGGGACAAACA (dTdT)
GFP	GCAAGCUGACCCUGAAGUUCAU

3.1.10 Cells

Cells indicated to carry viral RNA were transfected with a SGR of the given genotype. Cells without reference were produced by AG Lohmann.

Cell line	HCV genotype	selection	details
Huh-7	-	-	human hepatoma cell line[29]
Huh-7 JFH1	HCV 2a	1 mg/ml G418	described in[32]
Huh-7 Con1	HCV 1b	1 mg/ml G418	described as Huh-7 9-13 in[28]
Huh-7cured Con1	-	-	cured by IFN treatment as described in [105]
Huh-7 Con1 S2204R	HCV 1b	1 mg/ml G418	mutation affects replication efficiency
Huh-7 Con1 K1846T	HCV 1b	1 mg/ml G418	mutation affects replication efficiency
Huh-7 Con1 E1202G+ T1280S+ K1846T	HCV 1b	1 mg/ml G418	mutation affects replication efficiency
Huh-7 DV	-	75 µg/ml Hygromycin B	described in[106]
Huh-7 HAV	-	2.5 µg/ml of Blasticidin	described in[107]
Huh-7 Luc JFH	HCV 2a	1 mg/ml G418	viral RNA carries Luciferase gene[33]
Huh-6	-	-	human hepatoblastoma cell[108]
Huh-6 Con1	HCV 1b	0.5 mg/ml G418	described in[109]
Huh-6 JFH	HCV 2a	0.5 mg/ml G418	A. Cerwenka (DKFZ, Heidelberg)
Huh-6 cured	-	-	cured by IFN treatment,

JFH			A. Cerwenka (DKFZ, Heidelberg)
Huh-7.5	-	-	described in [110]
Hep56D	-	-	murine hepatoma cell line[111]
Hep56D JFH1	HCV 2a	0.5 mg/ml G418, 5 µg/ml Blasticidin	

3.1.11 Mouse strains

Wildtype C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, USA). TLR7 KO mice were obtained from Stefan Bauer (University Marburg). Unc93b1 mutant mice harboring an H412R missense mutation[37] were received from the Max Planck Institute (Freiburg). Mice were bred under specific pathogen free (SPF) conditions. Breeding and monthly control for infections was performed according to the FELASA and GV-SOLAS guidelines 2014. Killing and dissection of mice was approved by the local authorities (Regierungspräsidium Karlsruhe) and experiments were properly recorded.

Mice were hold in small groups of 2-8 animals (Makrolon cages of type II and III) under SPF (specific pathogen free) conditions. They were kept in air-conditioned rooms (20-22°C) with a day-night cycle of 12 h each. Water and standardized food (pellet food Rod18 from Las Vendi) were available ad libitum. All mice were put in new cages at least once per week. Female mice with new-born pups were put in new cages twice per week after pups were one week old. Additionally, pelleted breeding food was applied to the the bottom of the cages.

3.1.12 Software

Software	Manufaturer
FACSDiva™	BD Bioscience, San Diego
GraphPad Prism version 6.00	GraphPad Software Inc., San Diego, USA
IDEAS version 6.0	Amnis, Merck Millipore, Darmstadt
ImageJ	National Institutes of Health, Bethesda, USA
Inkscape 0.48	Free Software Foundation Inc., Boston, USA
Magellan (ELISA reader)	Tecan, Salzburg, Austria
NanoDrop 3.0.1	Nanodrop Technologies, Rockland, USA
Perseus	Jürgen Cox, MPI of Biochemistry, Martinsried
R	R Foundation for Statistical Computing, Vienna, Austria)
StepOne™ Software v2.1	Applied Biosystems, Darmstadt

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Cell lines

All cells (see 3.1.10) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin antibiotics, 2 mM L-glutamine and non-essential amino acids. They were maintained in 15 cm cell culture dishes in an incubator at 37°C with 5% CO2 and 95% humidity. Splitting was done twice per week when confluence reached approximately 80%. Therefore, cells were washed once with PBS and then detached by trypsin / EDTA treatment for approximately 3 minutes. Trypsin was inactivated by addition of medium containing FCS and cells were seeded for passaging and experiments. In general cells were passaged 1:3 or 1:5 depending on further usage after 3 or after 4 days.

3.2.1.2 Isolation of immune cells from human blood

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of voluntary healthy donors (as approved by the local Ethic Committee Heidelberg, admission number: S-157/2006) by density gradient centrifugation. 15 ml Pancoll (density 1.077 g/ml) were pipetted in 50 ml Falcons tubes, blood was mixed with equal volume of PBS and carefully layered on top of the pancoll. After centrifugation at 1000 g for 20 min at 4°C without deceleration, the interphase (white ring of PBMCs) was collected and transferred into fresh 50 ml Falcon tubes. Collected cells from two different Falcon tubes were pooled in one fresh Falcon tube, filled up to 50 ml with PBS and centrifuged at 365 g for 10 min at 4°C. Supernatant was discarded, cells were resuspended in 50 ml PBS and centrifuged at 300 g for 15 min at 4°C. Again, supernatant was discarded and cells were now resuspended in 10 ml PBS and pooled in one tube. PBS was added to a volume of 50 ml and the cell suspension was centrifuged at 365 g for 10 min at 4°C. Supernatant was discarded and cells were resuspended in 10 ml RPMI supplemented with 10% heat inactivated FCS. Cell titer was determined by counting cells in a Neubauer chamber after diluting the cells in Trypan blue. For some experiments $CD14^+$ monocytes, $CD56^+$ NK cells or $CD304^+$ pDCs were purified or depleted by a positive selection kit according to the manufacturer's instructions with an AutoMACS separator (magnetic activated cell sorting). After PBMC

purification and counting a determined number of PBMCs was centrifuged at 300 g for 10 min at 4°C. Cells were resuspended in the provided MACS assay buffer and microbeads with specific antibodies against the respective cell surface markers were added. Volumes of assay buffer and microbeads were used as instructed by the manufacturer dependent on the number of PBMCs used. After 15 min incubation at 4°C, 40 ml PBS was added and centrifuged at 300 g for 10 min at 4°C. Cells were resuspended in 2 ml PBS and separation was performed with the AutoMACS separator with the program suggested by the manufacturer. After purification, 10 ml medium was added to the cells and cells were centrifuged at 300 g for 10 min at 4°C. Subsequently, medium was removed, cells were resuspended in 0.5 - 2 ml of medium, counted and plated as indicated in the specific experiments.

3.2.1.3 Generation of murine bone marrow derived macrophages (BMDM) and bone marrow derived dendritic cells (BMDC)

Bone marrow cells were isolated from femora and tibiae of mice that were sacrificed by CO_2 asphyxia. Bones were dissected and cut open at the ends. By using a 27 G needle and a 5 ml syringe bone marrow cells were flushed into a Falcon tube with the respective medium (see below). The cell suspension was centrifuged at 300 g for 7 min and the pellet was resuspended in 48 ml of medium. For differentiation into BMDM, 12 ml of bone marrow cells were seeded per 15-cm Petri dish in DMEM (10% FCS, and 1% penicillin/streptomycin) supplemented with 6 ml supernatant of L929 cells. Supernatant from L929 cells contains macrophage colony-stimulating factor (M-CSF) that leads to differentiation of bone marrow cells into macrophages[112]. At day 4 after seeding the same amount of L929 supernatant was added. After 7 days differentiated macrophages were harvested. Cells were washed with PBS and then scraped in DMEM supplemented with 10% FCS. For generation of BMDCs, cells were seeded into 15-cm cell culture plates in RPMI 1640 supplemented with 4% LGM supernatant (containing GM-CSF), 10% FCS, 1% penicillin/streptomycin and 0.05 mM 2-ME. At day 4 fresh LGM supernatant was added and at day 7 non-adherent dendritic cells were harvested by centrifugation at 300 g for 10 min. BMDC were then resuspended in RPMI supplemented with 10% FCS.

3.2.1.4 Co-culture setup

For experiments where Huh cells were co-cultured with PBMCs, 1 x 10^5 Huh cells were seeded in 48-well plates in 200 µl DMEM. Following adherence of Huh cells (approximately 4 h) medium was removed and 1 x 10^6 PBMCs were added in 200 µl RPMI, as PBMCs did not survive in DMEM. After overnight co-culture supernatant or cells were harvested for further analysis. In experiments where purified subsets of specific immune cells were used for co-culture with Huh cells, 1 x 10^4 pDCs, 1 x 10^5 NK cells or 1 x 10^5 monocytes were used. Accordingly, when these cells were depleted from PBMCs, those numbers were subtracted from 1 x 10^6 PBMCs. These numbers approximately resemble the number of each subset of innate immune cells in 1 x 10^6 PBMCs.

In triple co-culture experiments 5 x 10^4 Huh-7 cells were seeded together with 5 x 10^4 Huh-7 Con1 cells and 1 x 10^6 PBMCs.

Co-culture experiments with murine cells were performed similarly. Here, 1 x 10^5 Hep56D cells were co-cultured with 3 x 10^5 BMDMs.

3.2.1.5 Transwell setup

Transwell inserts with a pore size of 1 µm were used to separate Huh-7 Con1 cells from PBMCs in the co-culture. For these experiments 1 x 10^5 Huh-7 Con1 cells and 1x10^6 PBMCs were used in a 24-well plate with a total volume of 500 µl (350 µl bottom well, 150 µl top well). Huh-7 Con1 cells were either seeded in the bottom or the top well in DMEM. After 4 h medium was removed and replaced by RPMI with 10% FCS. Along with that PBMCs were seeded in the opposite well also in RPMI with 10% FCS. For the setup of the invers transwell, the transwell inserts were placed in a sterile petri dish upside down. Thereby, Huh-7 Con1 cells were coated on the bottom of the transwell. After 4 h incubation medium was removed and transwell inserts were then placed into a 24 well plate and PBMCs were added in the top well. 22h after addition of PBMCs cell-free supernatants from both wells were collected and analyzed for IFNα levels by ELISA.

3.2.1.6 CFSE and CytoRED staining of Huh cells

In certain experiments Huh cells were labeled for flow cytometry analysis. Therefore, Huh cells were stained with CFSE or with Cytotell^{TM} RED (CytoRED) prior to seeding. A certain

amount of cells was centrifuged at 300 g for 7 min, resuspended in 1 ml PBS and incubated with the intracellular dye CFSE (4.5 µM in PBS) for 5 min at room temperature in the dark. Afterwards, cells were washed once with 10 ml PBS, once with 10 ml DMEM and then were seeded as described above. With exactly the same protocol Huh cells were stained with CytoRED for triple co-culture experiments. However, instead of staining cells with a 1x dye working solution of CytoRED as instructed by the manufacturer, cells were incubated with a 0.5x dye working solution to allow flow cytometry analysis (cells stained with 1x dye working solution were too bright).

3.2.1.7 Infection of Huh-7.5 cells with HCV

For HCV infection of Huh-7.5 cells the JC1 virus was used[113]. 5 x 10^4 Huh-7.5 cells were seeded in a 24-well plate in 500µl. After 24 h incubation, infection with the JC1 virus was performed for 6 h (MOI 3). Cells were then washed and stained with CFSE as described above inside the well. After 72 h, PBMCs were added to infected Huh-7 cells for overnight co-culture. Then cells were prepared for flow cytometry (see 3.2.2.3) and fixed with 1.5% PFA for 15 min at room temperature to allow further analysis under BSL1 conditions.

3.2.1.8 Stimulation of cells with recombinant cytokines

To analyze the effects of interferons on the uptake of Huh-7 cells by purified monocytes, these cells were co-cultured in the presence of 700 pg/ml IFNα or 7 ng/ml IFNγ. Equal amounts of IFNα were used in pre-stimulation experiments of either Huh-7 cells or purified monocytes. These amounts of cytokines were used as similar levels were measured in the co-culture of Huh-7 Con1 cells with PBMCs.

Sensitivity of Huh-7 cells was analyzed by TRAIL and TNFα stimulation. Huh-7 cells were treated with 50 or 100 ng/ml of TRAIL or TNFα for different time points as indicated in the figure legends.

3.2.1.9 Blocking antibodies

In different sets of experiments blocking antibodies against CD81, CD9 or TRAIL and the appropriate IgG control antibodies were used. In these experiments, co-cultures were set

up as described before (see 3.2.1.4). Antibodies were added to PBMCs directly before addition of PBMCs to Huh-7 cells. The concentrations used are given in the respective figure legends.

Experiments were performed to analyze the effect of IFNα on viral replication. Therefore, conditioned supernatant from the co-culture of HCV SGR cells with PBMCs was harvested. The conditioned supernatant was treated with an IFNα blocking antibody or an appropriate IgG control antibody for 1h in different dilutions and was then added to Huh-7 Luc JFH cells. Stock concentrations of both antibodies were 0.5 mg/ml. Dilutions used are given in the respective figure legends.

3.2.1.10 Exosome purification

Exosomes were isolated from supernatants of Huh-7 cells by subsequent centrifugation steps. Supernatants from 6 15 cm cell culture plates were pooled after 3 days of seeding. First, the supernatant was centrifuged at 300 g for 10 min at 4° C to remove cell debris. The pellet was discarded, and the supernatant again cleared by centrifugation at 2000 g for 20 min at 4°C followed by centrifugation at 10,000 g for 30 min at 4°C. The cleared supernatant was then used for ultracentrifugation at 100,000 g (28,000 rpm with a SW28 rotor) for 4 hours at 4° C. The supernatant was then discarded and the pellet containing exosomes was resuspended in PBS.

3.2.2 Immunoassays

3.2.2.1 Enzyme-linked immunosorbent assay (ELISA)

Cytokine secretion was quantified by Sandwich-ELISA. Cell-free supernatants were harvested and cytokine levels were measured according to the manufacturer's instructions. However, ELISAs were performed in half area plates. Accordingly, the volumes were adjusted to this setup. For detection of IFNα and murine IL-6 pure samples were used, while for IFNγ samples were diluted from 1:2 to 1:10 in ELISA blocking buffer. Samples that were stimulated by R848, CpG-DNA or LPS were diluted 1:10. Measurements were performed with the Sunrise plate reader and standard curves were plotted by the software Tecan Magellan.

3.2.2.2 Luminex™ assay

Next to cytokine quantification by ELISA, in cooperation with Prof. Dr. Christine Falk (Hannover) a Luminex™ assay was performed. Luminex™ allows the detection of multiple cytokines simultaneously within the same sample. Therefore, beads coated with different cytokine-specific antibodies are used. Moreover, beads are color-coded which allows an assignment of each bead to a specific cytokine. Beads are incubated with the sample and captured cytokines are then detected by biotinylated detection antibodies. A PE-streptavidin-conjugate is added to the samples and the beads are then analyzed by a dual laser instrument. While one laser is used for differentiation of the beads based on their color code, the other laser measures the PE signal which correlates with the amount of cytokine in the sample. A standard curve for each cytokine allows quantification.

For Luminex analysis undiluted supernatants of 4 independent experiments were sent to Prof. Dr. Christine Falk. Supernatants were collected from Huh-7 cells, PBMCs, Huh-7 Con1 cells and Huh-7 JFH cells cultured alone and from the co-culture of Huh-7 cells with PBMCs, of Huh-7 Con1 cells with PBMCs and of Huh-7 JFH cells with PBMCs. Cell numbers and volumes were used as described in 3.2.1.4. Also Huh-7 cells cultured alone were kept in RPMI from the time point on where co-cultures were set up.

3.2.2.3 Flow cytometry

To check for purity of purified immune cells (or for depletion efficiency) $2x10^5$ isolated cells were pipetted into FACS-tubes and mixed with 500 µl of FACS buffer. Samples were then centrifuged at 300 g for 5 min at 4°C and supernatant was discarded afterwards. Cells were resuspended in 100 µl FACS buffer, antibodies were added (in the dilution suggested by the manufacturer) and shortly vortexed. As negative controls samples were included without addition of the antibody. After 15 min at 4°C in the dark, 1 ml FACS buffer was added, shortly vortexed and centrifuged as before. Supernatant was discarded and cells were resuspended in 300 µl FACS buffer. FACS analysis was performed with a BD FACS Canto. If necessary, compensation was performed using single stains with the BD Diva™ software.

In experiments where Huh-7 cells and PBMCs (or purified monocytes) were co-cultured, cells were harvested for flow cytometry by the following procedure. Supernatants containing suspension cells were transferred to FACS tubes. Adherent cells were washed

with PBS, Trypsin/EDTA was added and incubated for 3 min at 37°C. Cells were resuspended carefully to obtain single cells and transferred to the same FACS tubes as the supernatant before.

For intracellular staining of IL-8, co-cultures of Huh-7 cells and PBMCs were set up as described. To mark Huh-7 cells, they were stained with CFSE prior to seeding. Brefeldin A (1 µg/ml) was added 14 h after PBMC addition to block cytokine secretion. After additional 6 h, cells were harvested as described before and washed with FACS buffer (centrifuged at 365 g for 5 min at 4°C). Monocytes were stained with CD14-PE as described above. After another wash, cells were fixed by addition of 250 µl 1.5% PFA under mild vortexing. Samples were incubated for 15 min at room temperature and washed twice with FACS buffer. Pellets were re-suspended in 500 µl permeabilization buffer and incubated for 5 min at 4°C. Cells were centrifuged, resuspended in 100 µl permeabilization buffers and incubated with anti-IL-8-APC antibody for 20 min at 4°C in the dark. Afterwards, cells were washed, resuspended in 300 µl FACS buffer and analyzed with the BD FACS Canto. Unstained controls as well as Huh-7 cells and Huh-7 Con1 cells seeded alone were used to determine background fluorescence.

In order to investigate uptake by monocytes, CFSE (or CytoRED) labeled Huh cells were co-cultured with PBMCs or purified monocytes as described. After harvesting (see above), cells were washed with FACS buffer and centrifuged at 365 g for 5 min at 4°C. Cells were resuspended in 100 µl FACS buffer and stained with CD14-PE as described. Cells were washed, resuspended in 300 µl FACS buffer and analyzed with the BD FACS Canto. CFSE signal was detected in the FITC channel, CytoRED in the APC channel. Single stains were used for compensation of the overlapping emission spectrum for CFSE and the PE channel.

Detection of the activation markers CD80 and CD86 on monocytes was performed by staining monocytes with CD14-APC and with CD80-PE or CD86-PE antibodies. NK cells activation markers CD69 and CD25 as well as TRAIL were analyzed on NK cells ($CD56^+$ and $CD3^-$) by the following stainings:

CD25-PerCP-Cy5.5, CD56-APC and CD3-FITC.

CD69-APC, CD56-Pe-Cy7 and CD3-FITC

TRAIL-PE, CD56-APC and CD3-APC-Cy7

3.2.2.4 ImageStream analysis

ImageStream technology is a combination of flow cytometry and microscopy. A picture is taken of every cell that runs through the flow chamber, thereby giving detailed information about the localization of staining detected by flow cytometry. For ImageStream analysis CFSE-stained Huh-7 cells were co-cultured with PBMCs overnight. Cells were harvested in the same procedure as described before for flow cytometry. After staining with CD14-PE, cells were fixed by addition of 250 µl 1.5% PFA under mild vortexing. Samples were incubated for 15 min at room temperature and washed twice with FACS buffer. Pellets were re-suspended in 500 µl permeabilization buffer and incubated for 5 min at 4°C. Cells were washed and then stained with Hoechst (1:10000) for 15 min at 4°C to stain nuclei. After another wash cells were resuspended in PBS and subjected to ImageStream analysis by PD Dr. Guido Wabnitz (Heidelberg). Measurements were recorded using an IS100 ImageStream device and analyzed with the software IDEAS version 6.0. For detection of the uptake of Huh-7 cells by monocytes, gates were set to detect $CD14^+$ monocytes that were also positive for CFSE. Microscopy pictures of these cells were then depicted.

3.2.3 Molecular Biology

3.2.3.1 Quantitative real time PCR (qPCR)

To quantify the amount of viral RNA qPCR was performed. RNA from Huh cells and from the supernatant of the cells was isolated with the NucleoSpin® RNA kit according to the protocol. In addition, RNA lysis buffer was spiked with an in-vitro transcript of Firefly Luciferase 2 (FL2). Thereby, a defined amount of RNA is present in each sample, which allows a normalization of viral RNA levels. Especially for quantification of viral RNA in the supernatant this is necessary, as RNA of standard housekeeping genes is not present in the supernatant. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions. As a negative control one sample was pipetted without reverse transcriptase (noRT). qPCR was then conducted with the SYBR Green Master Mix, which was mixed with water, cDNA (1:4 diluted before) and HCV specific primers to a final volume of 20 µl in a Micro Amp Fast 96-well Reaction plate (0.1ml). Water and noRT samples served as negative controls. The plate was covered with an Optical Adhesive Cover, centrifuged shortly and subjected to analysis in the Real Time

PCR System Step-One-Plus (Program setup: 95 °C 15 min; 40x [95°C 3 sec; 55°C 1 min, 60°C 1min]). Relative expression of viral RNA was calculated by the ΔC_T compared to the FL2 expression (rE = 2^(- ΔC_T)).

Expression levels of cellular mRNA were quantified in a similar way. However, RNA was isolated with the PeqGold Total RNA Kit and expression was normalized to the housekeeping gene β-actin.

3.2.3.2 Small interfering RNA (siRNA) transfection

4 x 10^4 Huh cells were seeded per well in a 24-well plate. After overnight incubation, 6 pmol of siRNA were mixed with 1 µl of Lipofectamine RNAiMAX transfection reagent in 100 µl of OptiMEM and incubated for 5 minutes at room temperature. This mix was added to cells dropwise. Medium was exchanged 24 h after transfection together with addition of PBMCs. siRNA targeting GFP (which was not expressed in Huh-7 cells) was used as control.

3.2.3.3 Surface proteome analysis

In order to compare the surface proteins between Huh-7 and Huh-7 Con1 cells, the surface proteome of both cells was analyzed by mass spectrometry. To extract surface proteins the Pierce Cell Surface Protein Isolation Kit was used according to the manufacturer's instructions. In brief, surface proteins were labeled by EZ-Link™ Sulfo-NHS-SS-Biotin (a thiol-cleavable amine-reactive biotinylation reagent). Cells were lysed and biotinylated surface proteins were purified with NeutrAvidin Agarose. After several washing steps, 400µl SDS page sample buffer containing DTT was added to break the disulfide bonds which led to elution of the proteins. Eluted proteins were then subjected to mass spectrometry analysis which was performed by Dr. Robert Hardt in the core facility of the ZMBH Heidelberg. Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Gels were cut in 8 fractions, proteins were digested by trypsin treatment and analyzed by mass spectrometry (Q-Exactive HF MS). Analysis was performed by Perseus software.

3.2.4 Biochemistry

3.2.4.1 Western Blot

To harvest samples for Western Blotting, cells were washed once with PBS, lysed in 1x SDS PAGE sample buffer and incubated at 95°C for 10 min. Samples were loaded onto a 10% acrylamide gel and run in a vertical gel chamber at 80 V for approximately 2 hours. Separated proteins were transferred to a nitrocellulose membrane by semidry blotting at 1.75 mA/cm^2 for 1 hour and 15 minutes. After blotting Ponceau S was used to control for an efficient transfer of the proteins. Unspecific binding was then blocked by incubating the membranes in 5% dry milk in 1xTBST for 1 h at room temperature. Primary antibodies against cleaved PARP and ß-actin were diluted in blocking buffer 1:1000 and 1:5000, respectively. Membranes were incubated with primary antibody overnight at 4°C, washed three times for 10 min with 1xTBST at RT and incubated with HRP-conjugated anti rabbit antibody diluted 1:4000 in blocking buffer for 45 min at RT. Again, membranes were washed as described above and proteins were then detected by using enhanced chemiluminescence (ECL) substrate (1:1 mix of Reagent A and Reagent B added to the membrane). Densitometric analysis was performed using ImageJ software.

3.2.4.2 Luciferase assay

Huh-7 Luc JFH cells were seeded in 96-well plates as described above. After 4h, DMEM was removed and cells were incubated overnight with 200 µl conditioned supernatant (derived from co-culture of Huh-7 Con1 cells with PBMCs). Medium was removed and cells were lysed in 50 µl Luciferase lysis buffer. Plates were placed on a shaker for 15 min at RT and subsequently frozen at -80°C. After complete freezing, cells were thawed at RT and samples were analyzed with a plate luminometer. 300 µl of Luciferase assay buffer were injected into each well automatically, and light signal was measured for 10 seconds. 40 µl of a 10% SDS solution were injected to stop the light reaction. Luciferase activity in Huh-7 Luc JFH cells kept in fresh medium was used as a reference. The luciferase activity measured in these cells was set to 100%. Supernatants from PBMCS or from Huh-7 Con1 cells alone served as additional controls.

3.2.4.3 Lactate dehydrogenase (LDH) release assay

Release of LDH was used to quantitatively measure cytotoxicity using the Cytotoxicity Detection Kit Plus (LDH). Typically, LDH is an intracellular protein, but is released from cells during apoptosis or necrosis. Upon release of LDH, activity of the enzyme can be detected in the supernatant. Addition of an LDH substrate results in its conversion into a red-colored product causing colorimetric shift of cell culture supernatants, which is proportional to LDH content and cytotoxicity and can be measured by a photometer.

Measurement of extracellular LDH activity was determined according to the manufacturer's protocol, however, with half the volumes suggested. Huh cells were incubated with recombinant proteins or with PBMCs overnight. As a positive control (100% killed cells) 1 µl Tween-20 was added to Huh cells, carefully mixed and incubated for 30 min at 37°C. To determine background activity, each population of cells used in a specific experiment was cultured alone in an extra well. Cell culture supernatants were harvested in tubes and centrifuged at 300 g for 10 min at 4°C. 50 µl of cell-free supernatants were transferred to 96-well F-bottom plates and incubated with 50 µl of the reaction mixture for 30 min in the dark. Reaction was stopped by addition of 25 µl of the provided stop solution. Absorbance was read with the Sunrise ELISA reader at 490 nm.

To determine the percentage of killed Huh cells, culture medium background was subtracted from all values. Subsequently, the formula provided in the instructions was used to calculate the percentage of cytotoxicity in Huh cells: In detail, spontaneous cell death of target cells (Huh cells) and effector cells (e.g. PBMCs) were subtracted from experimental values of the co-culture, followed by normalization to maximum release from target cells (Huh cells lysed by Tween-20) subtracted by spontaneous cell death of target cells. This quotient was multiplied with 100 to obtain the percentage of cytotoxicity. All samples were set up as duplicates.

$$cytotoxicity\ (\%) = \frac{(PBMC + Huh7) - PBMC - Huh7}{Huh7\ (Tween) - Huh7} \times 100$$

3.2.5 Statistical analysis

All experiments were performed at least three times unless indicated otherwise in the figure legends. Error bars indicate the standard deviation (SD). For all experiments in which PBMCs

from different donors were used, it was analyzed by ANOVA whether the donor differences had a significant effect. In that case a linear mixed effect model was performed to add a random effect due to the donor variations. Thus, distribution of samples had no implications, as linear mixed effect models are robust against breaking the normality assumption. Finally, multiple comparisons were performed by Anova followed by a Tukey post-hoc test to analyze significant differences in different conditions. $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were considered significant. Non-significant differences were indicated by ns. All calculations were performed with R.

4 Results

4.1 pDC activation by Hepatitis C virus subgenomic replicon cells depends on viral RNA and cell-cell contacts

4.1.1 pDCs secrete IFNα in response to Hepatitis C virus subgenomic replicon cells

In order to analyze the response of innate immune cells against the Hepatitis C virus, at first an experimental approach had to be established. Previous studies had shown that the co-culture of Hepatitis C virus subgenomic replicon (HCV SGR) cells and human peripheral blood mononuclear cells (PBMCs) resulted in IFNα secretion by plasmacytoid dendritic cells (pDCs)[59]. Thus, a co-culture system was used here to further analyze the innate immune response against HCV. To establish the experimental setup IFNα secretion by PBMCs in co-culture with HCV SGR cells was analyzed. In preliminary experiments various parameters were tested. The time point of addition of PBMCs to HCV SGR cells as well as the number of SGR cells and PBMCs were optimized (data not shown) to obtain a reliable and reproducible activation of PBMCs. Of note, as this project focused on the role of viral RNA in PBMC activation, HCV SGR cells were used instead of HCV infected cells.

In this study the HCV SGR cell termed Huh-7 Con1 (HCV genotype 1b) was used. Naïve Huh-7 hepatoma cells as well as Huh-7 Con1 cells that were cured from the replicon ("cured Con1") served as controls for specific PBMC activation by HCV SGR cells. Indeed, IFNα was secreted in the co-culture of the HCV SGR cell line Huh-7 Con1 with PBMCs (Fig. 4-1 A). Neither Huh-7 nor cured Con-1 cells triggered IFNα production by PBMCs. To confirm that pDCs within the PBMC fraction produced IFNα, pDCs were depleted from PBMCs before addition to Huh-7 Con1 cells. After pDC depletion no IFNα was detected anymore (Fig. 4-1 B). In another approach purified pDCs were co-cultured with Huh-7 Con1 cells. Similar to complete PBMCs, in this setup IFNα was secreted (Fig. 4-1 C) proving that pDCs respond to HCV SGR cells with secretion of IFNα.

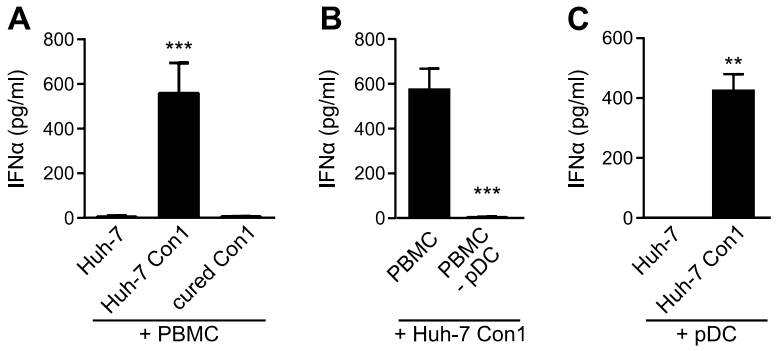

Fig. 4-1 pDCs secrete IFNα in response to Hepatitis C virus subgenomic replicon cells
(A) Huh-7 cells (Huh-7 has no HCV SGR, Huh-7 Con1 carries HCV SGR genotype 1b, cured Con1 were cured from the replicon by IFN treatment) were co-cultured with PBMCs overnight. Supernatants were collected and measured for IFNα secretion by ELISA. (B) IFNα levels in co-cultures with PBMCs or with PBMCs depleted from pDCs (PBMC -pDC). (C) IFNα levels in co-cultures with MACS-purified pDCs (n=3).

4.1.2 Secreted IFNα inhibits viral replication

Having observed that pDCs secreted IFNα in co-culture with HCV SGR cells, the question was addressed whether the amount of type I IFN was sufficient to exert an anti-viral effect. To analyze this, Huh-7 JFH (HCV genotype 2a) SGR cells that additionally encode a luciferase gene were used (Huh-7 Luc JFH). With these Huh-7 Luc JFH reporter cells viral replication can be measured in a Luciferase assay. Inhibition of replication reduces the amount of luciferase and thereby the luciferase activity is decreased.

Huh-7 Luc JFH reporter cells were incubated overnight with conditioned supernatant derived from the co-culture of Huh-7 Con1 cells and PBMCs. Conditioned supernatant significantly reduced the luciferase activity in a dose-dependent manner (Fig. 4-2 A). Remarkably, the anti-viral effect of the conditioned supernatant was even observed at high dilutions. For example a 1:500 dilution reduced luciferase activity by 94.7%. The calculated IC50 dilution was at 1:2231. According to ELISA data the conditioned supernatant comprised 330 pg/ml of IFNα, indicating that 0.15 pg/ml IFNα inhibited viral replication by 50% (assuming the effect is mostly caused by IFNα, but also other cytokines might also inhibit viral replication). 1:2 diluted supernatant from Huh-7 Con1 cells or PBMCs cultured alone had no influence on the luciferase activity. Noteworthy, as no IFNα could be detected in a 1:500 dilution by ELISA, the Luciferase assay served as a bioassay with higher sensitivity.

To confirm that the observed reduction in viral replication was caused by IFNα, experiments using IFNα blocking antibodies were done in which the conditioned

supernatant was incubated with the antibody prior to addition to Huh-7 Luc JFH reporter cells. Indeed, blocking IFNα significantly restored Luciferase activity compared to conditioned supernatant with IgG control antibodies at the respective concentrations (Fig. 4-2 B). With the highest concentration of IFNα blocking antibody luciferase activity was restored to 58% (medium control set to 100%). Thus, IFNα blocking was either not complete or not only IFNα but also other secreted cytokines by PBMCs affected viral replication. Nevertheless, the results clearly showed that activation of PBMCs in the co-culture with HCV SGR cells leads to secretion of bioactive IFNα, which in turn inhibits viral replication.

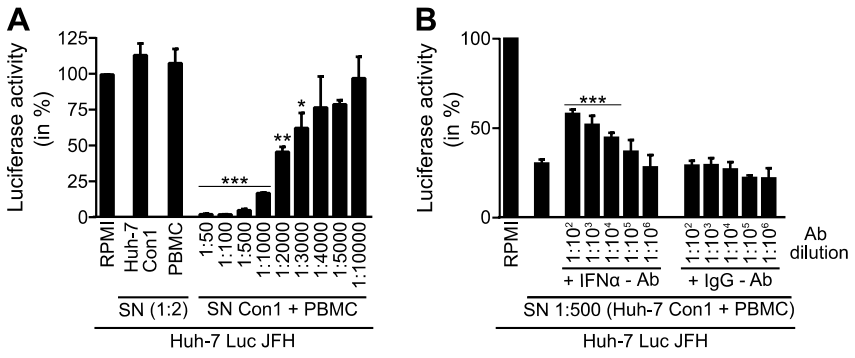

Fig. 4-2 pDC secreted IFNα inhibits viral replication
(A) Huh-7 Luc JFH cells were incubated overnight with different dilutions of conditioned supernatant (SN) from the co-culture of Huh-7 Con1 cells and PBMCs. Supernatants from Huh-7 Con1 cells and PBMCs alone were used as controls. After overnight incubation, supernatant was removed, cells were lysed in luciferase lysis buffer and luciferase activity was measured. Significance is indicated compared to RPMI control (n=3).
(B) Conditioned supernatant (SN) from the co-culture of Huh-7 Con1 cells and PBMCs was diluted 1:500 and pretreated with an IFNα or an IgG control antibody in the same concentrations for 1 h. Huh-7 Luc JFH cells were then incubated with these pretreated supernatants overnight. After overnight incubation experiments were conducted as in (A) Significance is indicated compared to IgG control antibodies (n=3).

4.1.3 pDCs are stimulated by viral RNA in co-culture with HCV SGR cells

Previous experiments showed that pDCs were activated in the co-culture with Huh-7 Con1 cells and that they secreted IFNα, which exerted an anti-viral activity. However, it was unclear what exactly the stimulus for pDC activation is. As a previous study provided evidence that viral RNA triggers pDC activation[59], this hypothesis was further investigated. Several SGR cells (Huh-7 JFH, Huh-7 Con1, Huh-7 Con1 mutants K1846T, S2204R and E1202G+T1280S+K1846T, Huh-6 Con1) were therefore tested: the different mutations in

the Con1 background influence the replication efficiency of the replicons (V. Lohmann, Dpt. of Molecular Virology, personal communication). Hence, pDCs would encounter different amounts of viral RNA in the co-culture. If viral RNA in fact is the stimulus for pDC activation, the difference in viral RNA levels should result in a different degree of pDC activation.

To analyze this, co-cultures of the various SGR cells with PBMCs were performed. pDC activation was measured by IFNα secretion and viral RNA was quantified in the supernatant of SGR cells. In three independent experiments a positive correlation between viral RNA in the supernatant and IFNα production by pDCs was observed (Fig. 4-3). For Huh-7 JFH and the Huh-7 Con1 E1202G+T1280S+K1846T mutant SGR cell only low amounts of viral RNA were detected in the supernatants. Accordingly, no or very low levels of IFNα were measured in the co-cultures with PBMCs. In contrast, supernatants of Huh-7 Con1 SGR cells showed high amounts of viral RNA. In consequence, high levels of IFNα were produced by pDCs in the co-culture. The other tested SGR cells showed intermediate RNA levels and in line with that intermediate IFNα production by pDCs.

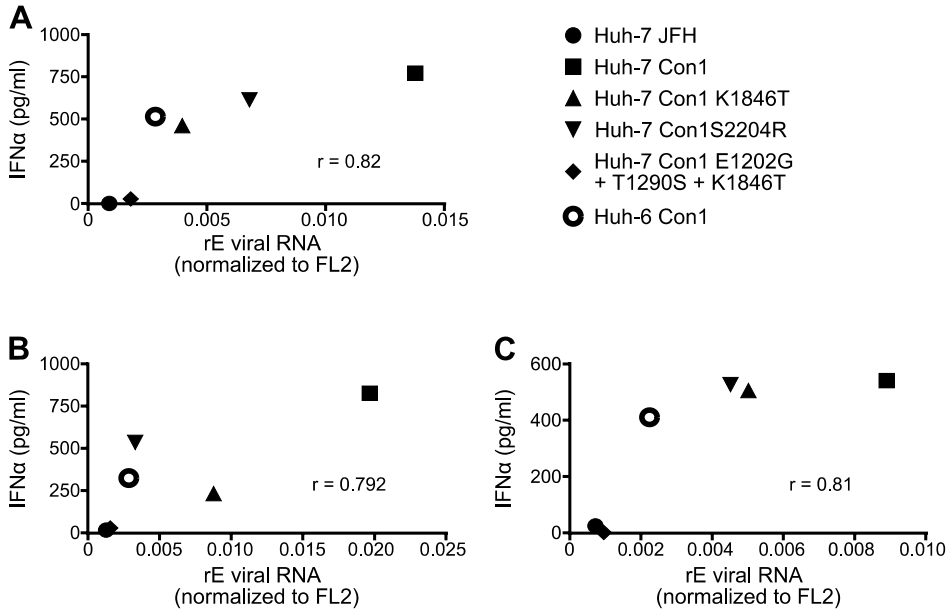

Fig. 4-3 IFNα secretion by pDCs correlates with secreted levels of viral RNA
Different SGR cells (Huh-7 JFH, Huh-7 Con1, Huh-7 Con1 mutants K1846T, S2204R and E1202G+T1280S+K1846T, Huh-6 Con1) were co-cultured with PBMCs overnight. Supernatants were collected and measured for IFNα level by ELISA. In parallel, only SGR cells were seeded for quantification of viral RNA in the supernatant by qPCR. RNA lysis buffer was spiked with a Firefly Luciferase 2 (FL2) *in vitro* transcript for normalization. Three independent experiments are shown (A, B and C). r = Pearson correlation coefficient.

4.1.4 Bafilomycin inhibits pDC activation in co-culture with HCV SGR cells

The observation that secretion of viral RNA from HCV SGR cells correlated with IFNα production by pDCs already indicated that viral RNA might be triggering pDC activation. Expression of the endosomal nucleic acid pattern recognition receptor TLR7 in pDCs allows recognition of viral RNA. The role of viral RNA in pDC activation was further investigated using Bafilomycin and Chloroquine which both inhibit endosomal acidification. Thereby, endosomal TLRs lose their function and TLR mediated nucleic acid recognition is inhibited[114,115], while cytosolic RNA sensors are known to be insensitive to Bafilomycin.

Addition of Bafilomycin resulted in complete inhibition of pDC activation in the co-culture of Huh-7 Con1 cells and PBMCs (Fig. 4-4 A). To control specificity of the drug, PBMCs were also stimulated with LPS (TLR4 agonist, not endosomal) in the presence of Bafilomycin. Since TLR4 signaling does not result in IFNα secretion, IL-6 secretion was used as readout. LPS stimulation of PBMCs was not influenced by Bafilomycin (Fig. 4-4 B). The effects observed with Bafilomycin were reproduced with Chloroquine. Chloroquine prevented pDC stimulation in co-culture with Huh-7 Con1 cells (Fig. 4-4 C), but had no impact on LPS stimulation (Fig. 4-4 D). Hence, pDC stimulation by Huh-7 Con1 cells was dependent on endosomal signaling, probably mediated by TLRs.

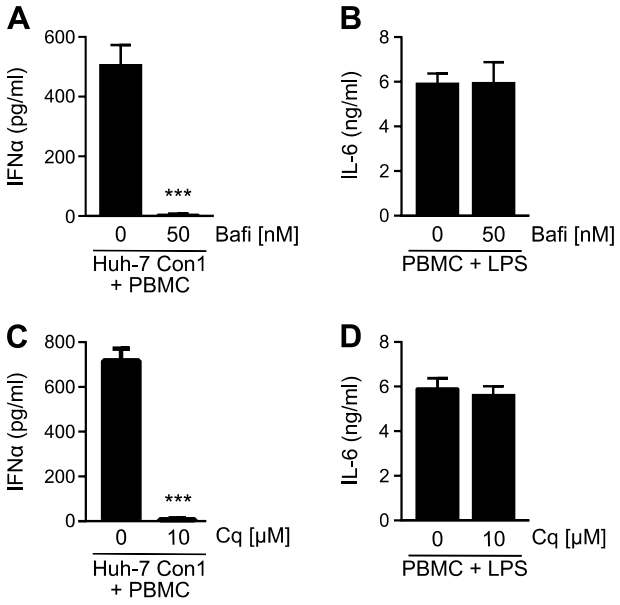

Fig. 4-4 Inhibition of endosomal TLRs affects pDC stimulation in co-culture with HCV SGR cells
(A, C) Huh-7 Con1 cells were co-cultured overnight with PBMCs that were pre-incubated with indicated concentrations of Bafilomycin (Bafi) or Chloroquine (Cq) for 1 h. Drugs were also present during co-culture period (n=3). (B, D) PBMCs were stimulated with LPS (0.1 ng/ml) under drug treatment (n=3). After overnight co-culture, supernatants were collected and measured for IFNα and IL-6 secretion by ELISA.

4.1.5 Response of murine bone marrow derived macrophages (BMDM) in co-culture with HCV SGR cells is partly dependent on TLR7 and fully dependent on endosomal TLR signaling

Experiments with human PBMCs revealed that endosomal recognition of viral RNA triggers pDC activation. To further investigate which TLRs contribute to the recognition of viral RNA, the co-culture setup was established in a murine system. This allowed the use of immune cells with defined deficiency for certain Toll-like receptors. The murine SGR cells Hep56D JFH were co-cultured with bone marrow derived macrophages (BMDM) and with bone marrow derived dendritic cells (BMDC) from wildtype mice. Interestingly, only BMDM, but not BMDC responded with IL-6 production in the co-culture with SGR cells (data not shown).

To analyze if recognition of viral RNA plays a role in the response of BMDM in the co-culture with SGR cells, BMDM from TLR7 KO mice were used, as TLR7 is known to recognize single stranded RNA. When comparing the stimulation of wildtype BMDM and TLR7 KO BMDM in the co-culture, it was observed that the production of IL-6 by TLR7 KO BMDM was significantly decreased (Fig. 4-5 A). TLR7 KO BMDM responded to the positive controls CpG-DNA (TLR9) and LPS (TLR4) in the same range as wildtype BMDM.

However, they did not respond to the TLR7 agonist R848, which proved that activation via TLR7 in these cells was not functional.

Although IL-6 levels were significantly lower in TLR7 KO BMDM, IL-6 was still detectable. Hence, in the next experiments mutant Unc93b1 mice were used. Unc93b1 is essential for trafficking of endosomal TLRs from the ER to endosomes[37]. Accordingly, in mutant Unc93b1 mice all endosomal TLRs are not functional and recognition of nucleic acids is prevented. Indeed, BMDM from mutant Unc93b1 mice were not stimulated at all by SGR cells, while wildtype BMDM produced IL-6 (Fig. 4-5 B). Upon LPS stimulation, which is recognized by TLR4 (not endosomal) mutant Unc93b1 BMDM and wildtype BMDM responded equally. In summary these results clearly showed that viral RNA is recognized by TLR7 with other endosomal TLRs also contributing. Thus, the experimental data support the previous finding of TLR mediated viral RNA recognition by human PBMCs.

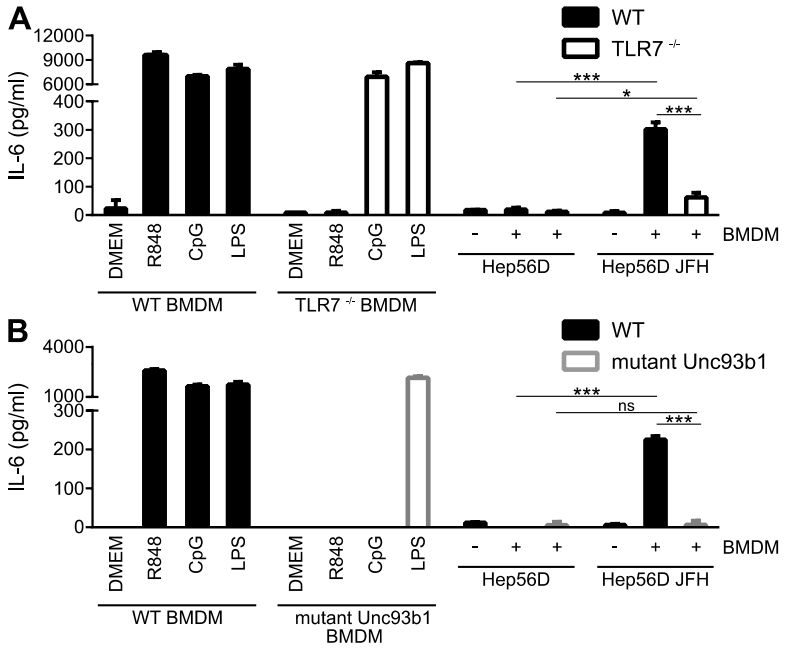

Fig. 4-5 Stimulation of BMDM in co-culture with murine HCV SGR cells is reduced in TLR7 KO mice and lost in mutant Unc93b1 mice
The murine HCV SGR cell Hep56D JFH was co-cultured with bone marrow derived macrophages (BMDM) from wildtype and TLR7 KO mice (A) or BMDM from mutant Unc93b1 mice (B) overnight. Supernatants were collected and measured for IL-6 levels by ELISA. R848 (1 µg/ml), CpG (1µM) and LPS (0.1 ng/ml) were used as positive controls (n=2).

4.1.6 Inhibition of exosome release reduces IFNα secretion

Having shown that viral RNA is stimulating pDCs, the next open question was how viral RNA is transported from HCV SGR cells into specialized immune cells that recognize the viral RNA as a PAMP and elicit an immune response. It has been published that viral RNA is secreted via exosomes and thereby transported to pDCs[63]. Thus, it was analyzed whether exosomes play a role in the stimulation of pDCs in co-culture with Huh-7 Con1 cells. Therefore, GW4869 and Spiroepoxide were used as exosome release inhibitors. Both inhibitors act via inhibition of neutral sphingomyelinases[116] but are structurally unrelated. If viral RNA in fact is transported via exosomes, an inhibition of exosome release would decrease the amount of secreted viral RNA. In turn, this should attenuate PBMC stimulation.

Thus, Huh-7 Con1 cells were treated with GW4869 or Spiroepoxide directly after seeding and over the entire period of co-culture with PBMCs. GW4869 significantly reduced the production of IFNα in co-culture with Huh-7 Con1 cells (Fig. 4-6 A). DMSO and GW4869 had no influence on R848 stimulation of PBMCs (Fig. 4-6 B), indicating that viability of pDCs was not affected by GW4869. However, the results obtained with GW4869 were not reproducible with Spiroepoxide: Spiroepoxide did not affect PBMC stimulation in co-culture with Huh-7 Con1 cells (Fig. 4-6 C).

Due to the inconsistent results with the two different exosome release inhibitors, in a third approach Rab27a was knocked down by siRNA. Rab27a is known to contribute to exosome release[117]. Knockdown of Rab27a significantly reduced IFNα secretion, but IFNα was still detectable in considerable amounts (Fig. 4-6 D), although the knockdown reduced Rab27a mRNA levels 83x fold (Fig. 4-6 E). In summary, exosome release inhibition experiments gave no clear answer, in how far viral RNA is transported to pDCs via exosomes.

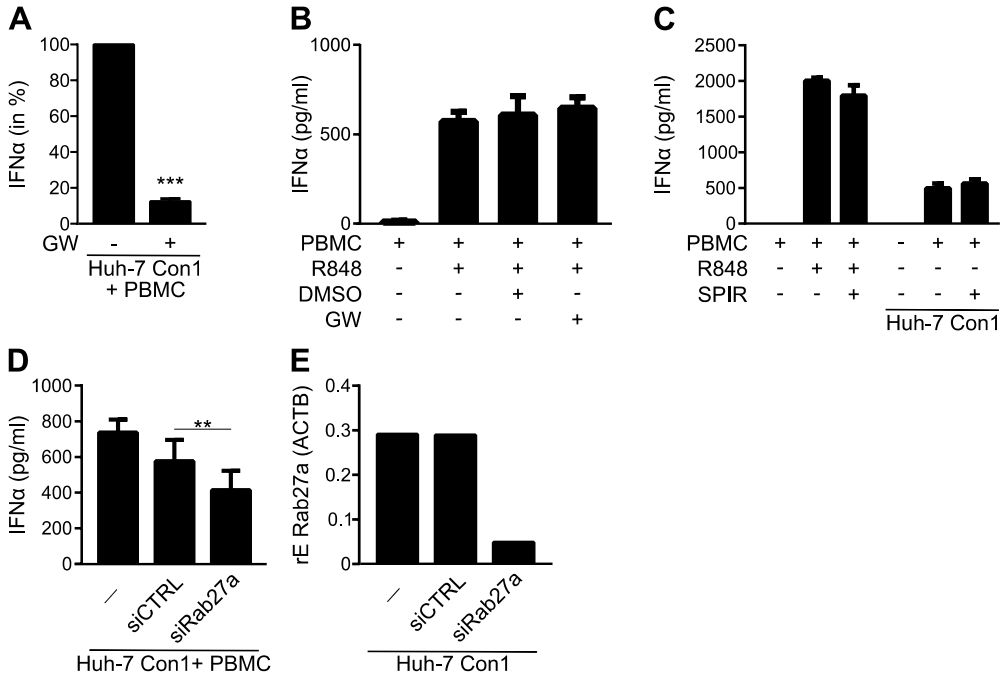

Fig. 4-6 pDC activation by HCV SGR cells is partly influenced by exosome release inhibition
Huh-7 Con1 cells were seeded in the presence of the exosome release inhibitors GW4869 (GW, 15 µM, (A) and (B)) or Spiroepoxide (SPIR, 5 µM, (C)). PBMCs were added 4 h after seeding and inhibitors were included also during co-culture. PBMCs stimulated with R848 (1 µg/ml) in the presence of inhibitors and DMSO were used as controls (B and C). (D) Huh-7 Con1 cells were treated with siRNA against Rab27a before addition of PBMCs. After overnight co-culture, supernatants were collected and measured for IFNα levels by ELISA. (E) qPCR analysis of Rab27a knockdown efficiency. n=5 for (A), n=2 for (B), n=4 for (C), n=3 for (D), n=1 for (E).

4.1.7 Purified exosomes from HCV SGR cells do not activate pDCs

In another approach to investigate the role of exosomes in transmission of viral RNA, exosomes were purified from HCV SGR cells and incubated with PBMCs. However, exosomes from Huh-7 Con1 cells did not cause IFNα secretion by pDCs (Fig. 4-7 A). Also the hypothesis that additional cell contacts of PBMCs to naïve Huh-7 or Huh-7 JFH cells (which by themselves did not significantly activate pDCs (Fig. 4-3)) together with exosomes from Huh-7 Con1 cells could cause stimulation of pDCs was proven wrong (Fig. 4-7 A). In numerous experiments purified exosomes did not trigger IFNα secretion. This was even more surprising when the amount of RNA was quantified. PBMCs cultured with purified exosomes from Huh-7 Con1 cells were exposed to 144x more viral RNA compared to the level of viral RNA in the supernatant of the direct co-culture with Huh-7 Con1 cells (Fig. 4-7 B). This led to the conclusion that purified exosomes are not sufficient to stimulate pDCs and that an alternative transport mechanism between HCV SGR cells and pDCs must exist.

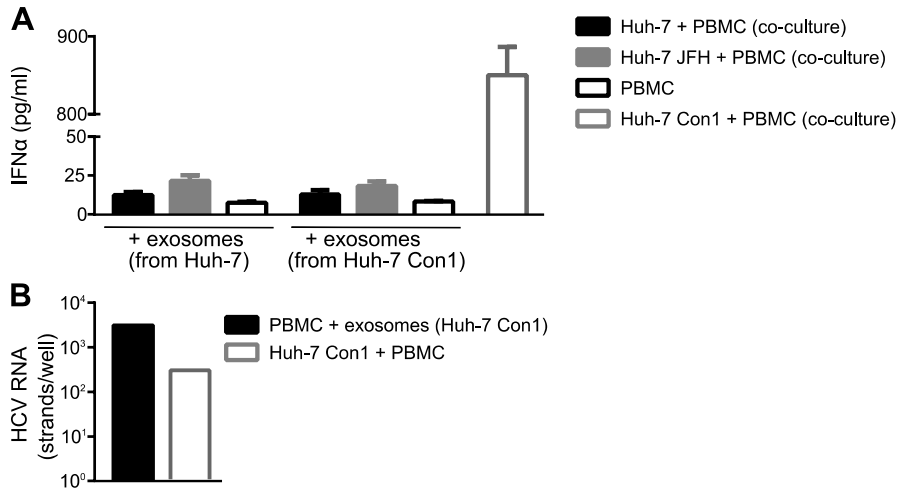

Fig. 4-7 Purified exosomes from HCV SGR cells do not activate pDCs
(A) Exosomes were purified from Huh-7 or Huh-7 Con1 cells and co-cultured with PBMCs alone or with PBMC together with Huh-7 or Huh-7 JFH cells overnight. Supernatants were collected and measured for IFNα secretion by ELISA (n=2). (B) Viral RNA content was determined by qPCR in purified exosomes and in the supernatant of Huh-7 Con1 cells (n=1).

4.1.8 Direct cell-cell contact between HCV SGR cells and pDCs is required for pDC activation

As purified exosomes did not stimulate pDCs, it was hypothesized that a direct contact between pDCs and HCV SGR cells is needed for transfer of viral RNA and subsequent pDC activation. To prevent direct cell-cell contact, PBMCs were separated from Huh-7 Con1 cells by a transwell (1µM pore size, which should allow diffusion of exosomes) in different setups resulting in physical separation with different distances. Independent of the position of PBMCs and Huh-7 Con1 cells no IFNα was secreted in transwell experiments (Fig. 4-8 A). In a second approach formation of cell-cell contacts was disturbed mechanically. Therefore, co-cultures were set up in parallel in two different plates. One of these plates was placed on an orbital shaker, while the other one was incubated without any movements. Afterwards pDC activation was analyzed by measuring IFNα in both conditions. PBMCs were also stimulated by R848 to control that shaking had no effect on PBMC viability and uptake of stimulating agents. In the co-culture of PBMCs with Huh-7 Con1 cells IFNα secretion was reduced by 83.4% when shaking (Fig. 4-8 B). R848 stimulation was not affected by shaking. These results indicated that tight cellular contacts between PBMCs and Huh-7 Con1 cells promote an efficient activation of pDCs.

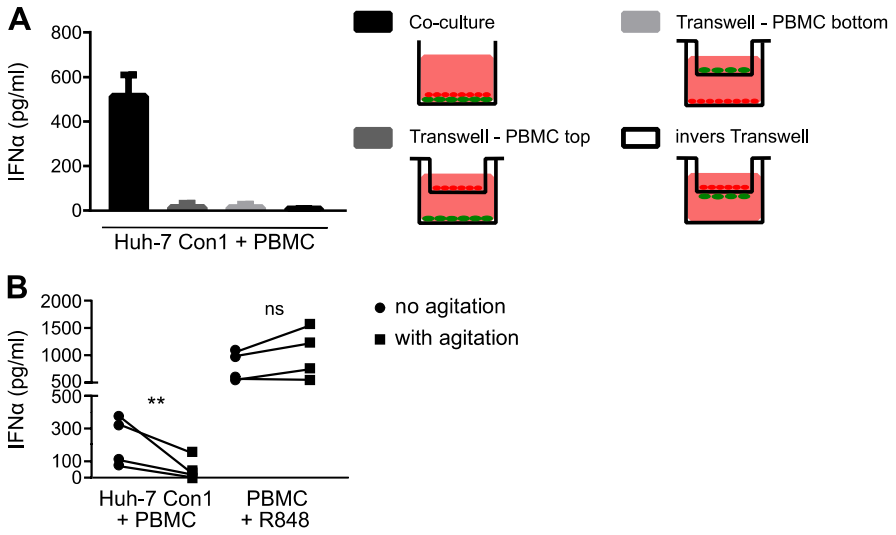

Fig. 4-8 Direct cell-cell contacts between HCV SGR cells and pDCs are required for pDC activation
(A) Huh-7 Con1 cells (depicted in green) were directly co-cultured with PBMCs (depicted in red) or separated by transwell (1µm pore size, membrane thickness 10 µm) as indicated. After overnight incubation, supernatants were collected and measured for IFNα secretion by ELISA (n=3). (B) Huh-7 Con1 cells were co-cultured with PBMCs either with or without mechanical agitation (orbital shaker, 200 rpm). After overnight co-culture, supernatants were collected and measured for IFNα levels by ELISA (n=4).

4.1.9 The tetraspanins CD81 and CD9 are involved in cell-cell contacts between HCV SGR cells and pDCs

Since previous experiments revealed a critical role of cell-cell contacts for pDC activation, in the next part it was analyzed how HCV SGR cells and pDCs interact with each other. An earlier study suggested a role of CD81 and CD9 in this interaction[118]. In consequence, it was analyzed if these surface proteins also played a role in the co-culture setup by using specific blocking antibodies. Indeed, blocking CD81 by antibodies significantly reduced IFNα secretion compared to IgG control antibodies. Similar results were observed when CD9 was blocked (Fig. 4-9). However, neither blocking of CD81 nor of CD9 completely prevented IFNα production, indicating that further interactions between HCV SGR cells and pDCs occur.

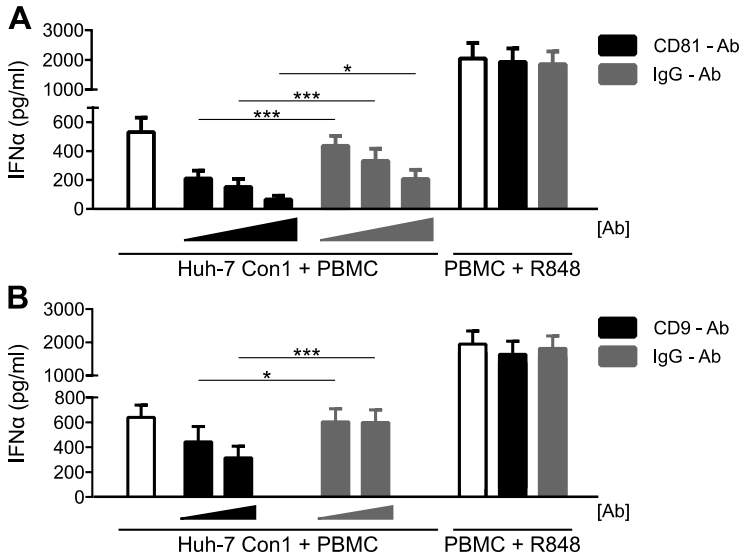

Fig. 4-9 CD81 and CD9 are involved in cell-cell contacts between HCV SGR cells and pDCs
Huh-7 Con1 cells were co-cultured with PBMCs in presence of CD81 (A, 0.1, 1, 2.5 µg/ml) or CD9 (B, 0.1, 1 µg/ml) blocking antibodies and respective IgG control antibodies. PBMCs stimulated with R848 (1 µg/ml) were incubated with the highest concentration of each antibody. After overnight co-culture, supernatants were collected and measured for IFNα levels by ELISA (n=4)

In summary, in this first part it was shown that pDCs respond to HCV SGR cells by IFNα secretion, which in turn inhibits viral replication. Furthermore, IFNα secretion was triggered by viral RNA which pDCs recognize by TLR7. For activation of pDCs direct cell-cell contacts with HCV SGR cells were necessary, most likely for an efficient transfer of viral RNA into pDCs, whereas activation by purified exosomes could not be observed. Nevertheless, it is possible that via direct contact the viral RNA is transported to pDCs via exosomes.

4.2 NK cells and monocytes respond to Hepatitis C Virus subgenomic replicon cells

4.2.1 PBMCs secrete a broad range of cytokines and chemokines in response to HCV SGR cells

Having characterized the IFNα response of pDCs against HCV SGR cells, it was investigated if further cytokines or chemokines are secreted by PBMCs in co-culture with HCV SGR cells. To this end a Luminex assay was performed in which 50 cytokines and chemokines were measured. Next to the co-culture of Huh-7 Con1 cells with PBMCs also supernatants from the co-culture of Huh-7 JFH cells with PBMCs were included, which

secrete less viral RNA and where no IFNα was detected in previous experiments (Fig. 4-3). The results of the Luminex assay are summarized in a heat map in Fig. 4-10. It was observed that several cytokines and chemokines were secreted in co-culture of PBMCs with Huh-7 Con1 cells as well as with Huh-7 JFH cells. The cytokine concentrations were mostly higher in Huh-7 Con1 co-cultures, indicating that these cells cause a stronger PBMC response. Nevertheless, also Huh-7 JFH cells activated PBMCs, despite the lack of IFNα secretion shown before.

In the following the cytokines and chemokines will be described that were detected in co-culture of Huh-7 Con1 cells and PBMCs. Pro-inflammatory cytokines IL-6 and TNFα were secreted. Besides IFNα (which confirmed previous ELISA data), also IFNγ was produced in the co-culture, indicating that also NK cells contribute to the response against HCV SGR cells. Moreover, several chemokines were detected that hinted towards a role of monocytes: IL-8, MCP1 (monocyte chemotactic protein 1), IP-10 (Interferon gamma-induced protein 10), MIP-1α (macrophage inflammatory protein 1-alpha), MIP-1β (macrophage inflammatory protein 1-beta). All of them can be secreted by monocytes, but also other cell types are known to produce these chemokines. In consequence, it was further analyzed in how far NK cells and monocytes play a role in the anti-viral response.

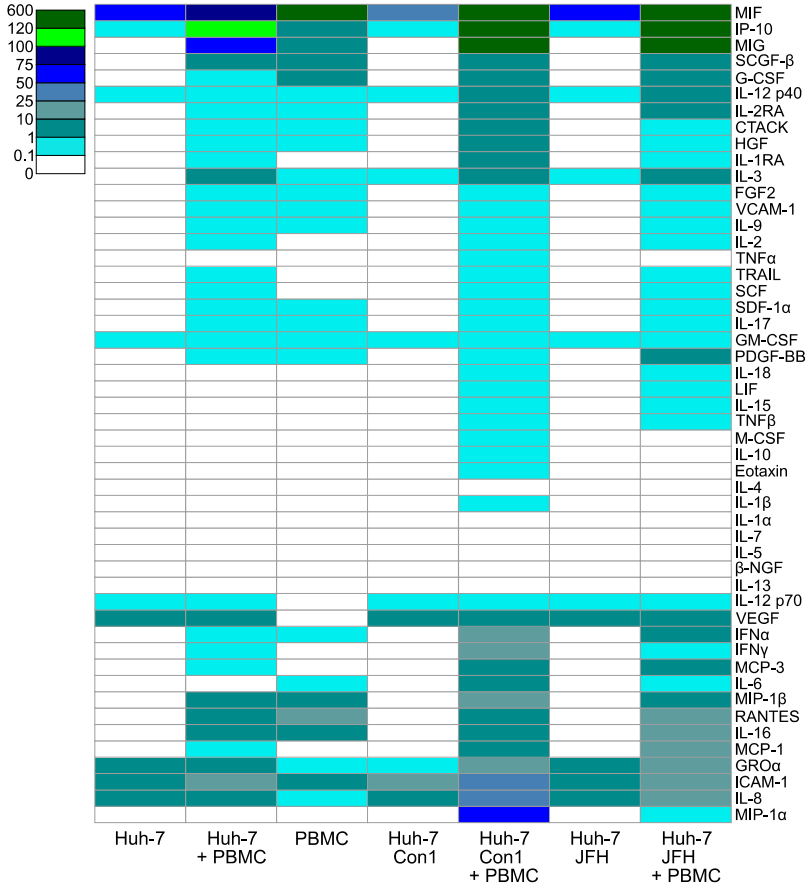

Fig. 4-10 PBMCs secrete a broad range of cytokines and chemokines in response to HCV SGR cells
Huh-7 cells (naïve, Con1 or JFH) were co-cultured with PBMCs overnight and the supernatant was analyzed by Luminex assay. Supernatant from different Huh-7 cells or PBMCs alone were used as controls (n=4). For quantification a standard curve for each cytokine was determined. Data are shown as the percentage of the highest standard of each cytokine.

4.2.2 NK cells secrete IFNγ in response to HCV SGR cells

Following the results of the Luminex assay, IFNγ secretion was also analyzed by ELISA. IFNγ was detected in the co-culture of the HCV SGR cell line Huh-7 Con1 with PBMCs (Fig. 4-11 A). Neither Huh-7 nor cured Con-1 cells triggered IFNγ production by PBMCs. To confirm that NK cells within the PBMC fraction produced IFNγ, NK cells were depleted from PBMCs before addition to Huh-7 Con1 cells. After NK cell depletion no IFNγ was detected anymore (Fig. 4-11 B). However, in contrast to pDC findings purified NK cells co-cultured with Huh-7 Con1 cells did not secrete IFNγ (Fig. 4-11 C), indicating that NK cells indirectly responded to HCV SGR cells.

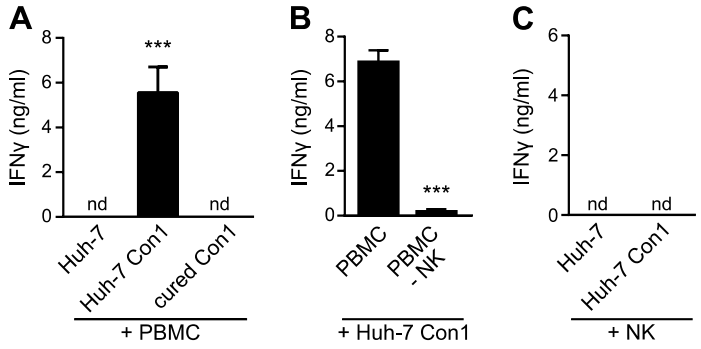

Fig. 4-11 NK cells secrete IFNγ in response to HCV SGR cells
(A) Huh-7 cells were co-cultured with PBMCs overnight and IFNγ was measured by ELISA. (B) IFNγ levels in co-cultures with PBMCs or with PBMCs depleted from NK cells (PBMC -NK). (C) IFNγ levels in co-cultures with purified NK cells (n=3). nd = not detected.

4.2.3 Validation of chemokine expression in response to HCV SGR cells

To confirm secretion of the chemokines detected in the Luminex assay (Fig. 4-12 A), expression of certain chemokines was additionally analyzed by qPCR. Compared to the co-culture of Huh-7 cells with PBMCs, all tested chemokines (IL-8, MCP-1, IP-10, MIP-1α and MIP-1β) were significantly up-regulated in co-cultures of Huh-7 Con1 cells with PBMCs on mRNA level (Fig. 4-12 B). These findings further confirmed that also monocytes might play a role in the response against HCV SGR cells.

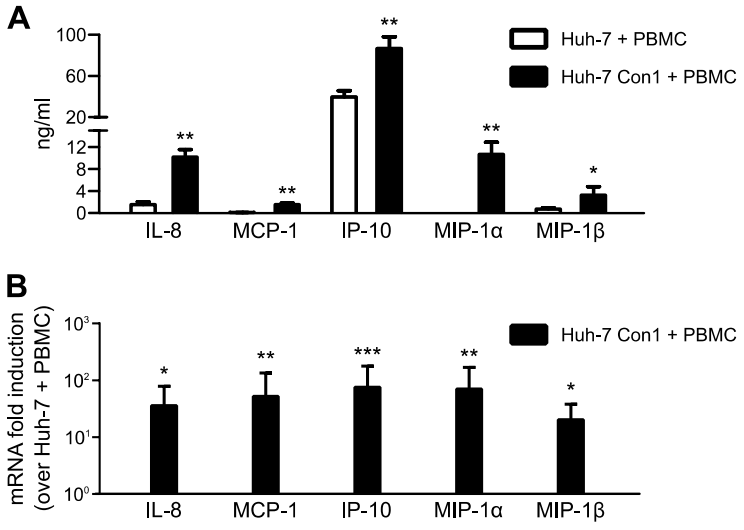

Fig. 4-12 PBMCs secrete multiple chemokines in response to HCV SGR cells
(A) Protein levels of selected chemokines from the Luminex assay are depicted (n=4). (B) Huh-7 (naïve or Con1) were co-cultured with PBMCs for 8h. RNA was extracted and qPCR was performed to quantify RNA levels of chemokines (n=3).

4.2.4 IL-8 is secreted by HCV SGR cells in co-culture with PBMCs

As shown before, several chemokines that can be secreted by monocytes were detected in the co-culture of Huh-7 Con1 cells with PBMCs. To identify if certain chemokines are secreted by monocytes or by HCV SGR cells, intracellular staining of IL-8 as one factor was done and analyzed by flow cytometry. Huh-7 cells were CFSE labeled prior to PBMC addition and monocytes were identified by CD14 expression. While in the co-culture of Huh-7 cells and PBMCs no IL-8 positive cells were detected (Fig. 4-13 A), 34% of Huh-7 Con1 cells became IL-8 positive in co-culture with PBMCs (Fig. 4-13 B). In contrast, only a very low percentage of monocytes showed IL-8 (not significant). Quantification of IL-8 positive Huh-7 cells as well as the MFI for IL-8 are shown in Fig. 4-13 C and D.

Taken together this observation demonstrates that IL-8 is not secreted by monocytes, but by Huh-7 Con1 cells. Apparently, anti-viral actions by PBMCs also cause a response within HCV SGR cells, which secrete the chemokine IL-8 to attract further immune cells. Nevertheless, (although not investigated) other chemokines might be secreted by monocytes. Importantly, this flow cytometry experiments by chance revealed another interesting observation about the role of monocytes, which will be described below.

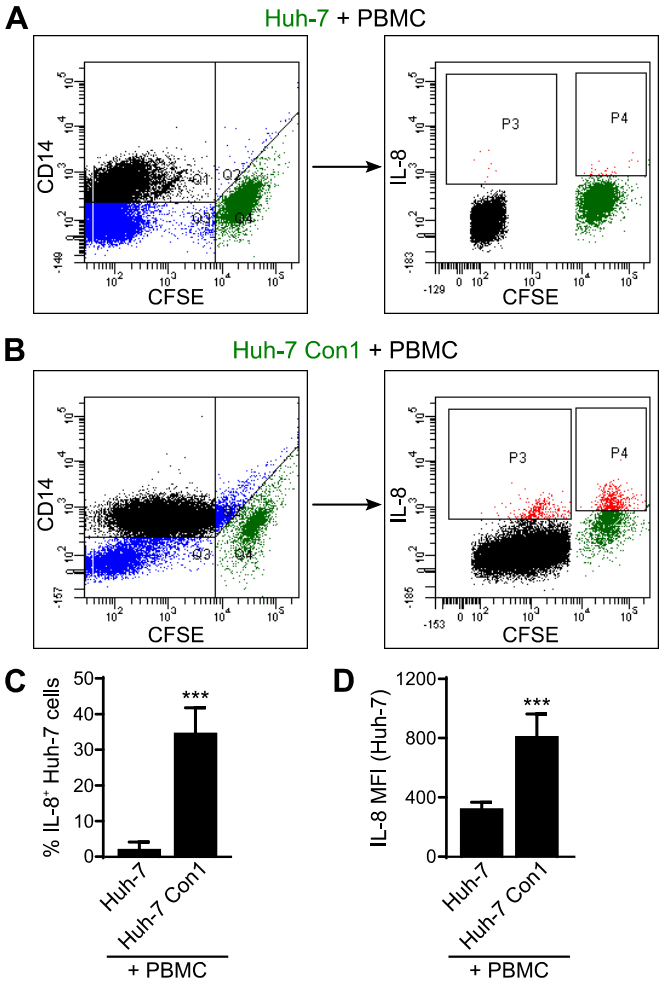

Fig. 4-13 IL-8 is secreted by HCV SGR cells in co-culture with PBMCs
Huh-7 cells (A) or Huh-7 Con1 cells (B) were stained with CFSE and co-cultured with PBMCs. After 14 h of co-culture, Brefeldin A (1 µg/ml) was added and cells were incubated for further 6 h. Monocytes were stained with a CD14 antibody; cells were fixed with 1.5% PFA and permeabilized using 0.1% Saponin. Intracellular IL-8 was stained with an IL-8 antibody and cells were analyzed by flow cytometry. In the left plots in (A) and (B) gates were set to identify monocytes (black) and Huh-7 cells (green). Both populations are depicted in the right plot to analyze which cells were positive for IL-8. Quantification of IL-8 positive Huh-7 cells (C) and the respective MFI (D) are shown (n=3). Plots from one representative experiment are shown in (A) and (B).

4.2.5 Monocytes take up HCV SGR cells

While monocytes did not secrete IL-8, it was observed that monocytes turned CFSE positive in co-culture of CFSE-stained Huh-7 Con1 cells with PBMCs (compare top left quadrant Fig. 4-13 A and B). This finding was further investigated: Huh-7 cells were stained with CFSE and then co-cultured with PBMCs. However, no Brefeldin was added in this set of experiments, as no intracellular staining was performed. After overnight co-culture with CFSE-stained Huh-7 cells, monocytes were CFSE negative (Fig. 4-14 A, left plot Q1). Strikingly, in the co-culture of CFSE-stained Huh-7 Con1 cells with PBMCs nearly all monocytes turned CFSE positive (Fig. 4-14, right plot Q2).

In order to analyze why monocytes became CFSE positive, ImageStream analysis was performed. ImageStream combines flow cytometry with microscopy, so that a picture is taken of every cell that is running through the flow chamber. Thereby, it became clear that monocytes take up small particles derived from Huh-7 Con1 cells (Fig. 4-14 B, lower panel, Fig. 4-15). In contrast, the few CFSE positive monocytes that appeared in the co-culture with Huh-7 cells did not internalize material from these cells but only were in close contact (Fig. 4-14 B, upper panel). The size of the particles taken up from Huh-7 Con1 cells ranged from 1.8-3.3 µm in diameter, suggesting that activated PBMCs induced apoptosis of HCV SGR cells. Induction of apoptosis was supported by the observation that the number of Huh-7 Con1 cells significantly dropped compared to the number of Huh-7 cells in co-culture with PBMCs (shown in Fig. 4-14 A Q4, quantified in Fig. 4-14 C). This was determined by calculating the ratio of Huh-7 cells and monocytes. At the start of the co-culture approximately 1.5×10^5 Huh-7 cells and 1×10^5 monocytes were present. While the number of Huh-7 control cells and monocytes remained constant, the number of Huh-7 Con1 cells clearly decreased. Thus the ratio of Huh-7 Con1 cells and monocytes significantly dropped.

In summary, these findings proved that monocytes are involved in the response against HCV SGR cells.

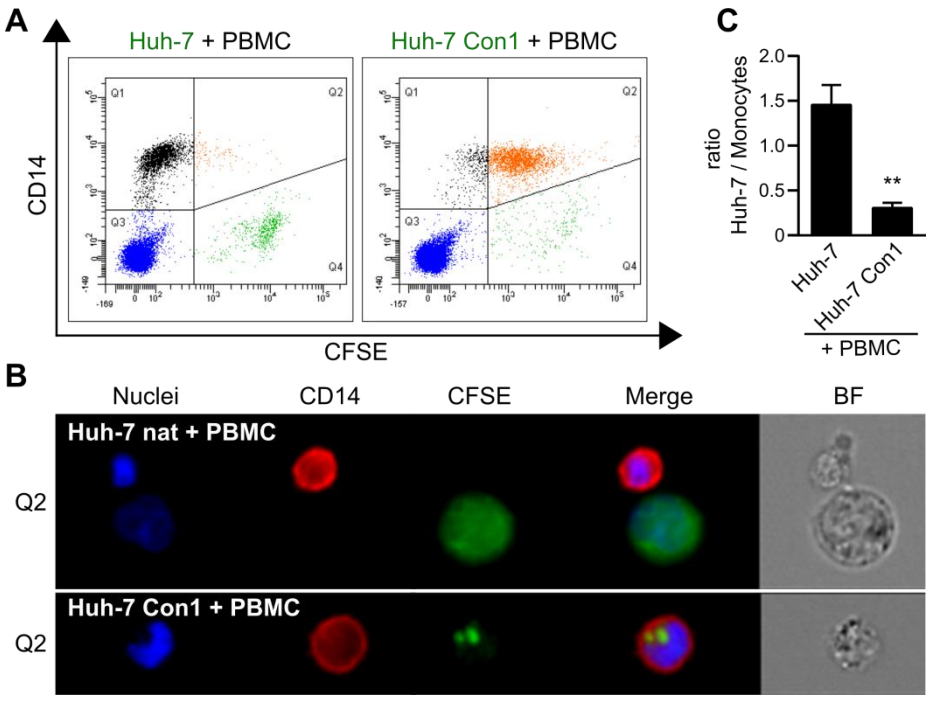

Fig. 4-14 Monocytes take up HCV SGR cells
CFSE-stained Huh-7 cells were co-cultured with PBMCs overnight. (A) Analysis of CFSE distribution in relation to monocytes ($CD14^+$). (B) Uptake of Huh-7 cells by monocytes depicted by ImageStream analysis. (C) Ratio between Huh-7 cells and monocytes after co-culture based on flow cytometry analysis in (A) (n=3). Plots from one representative experiment are shown in (A) and (B).

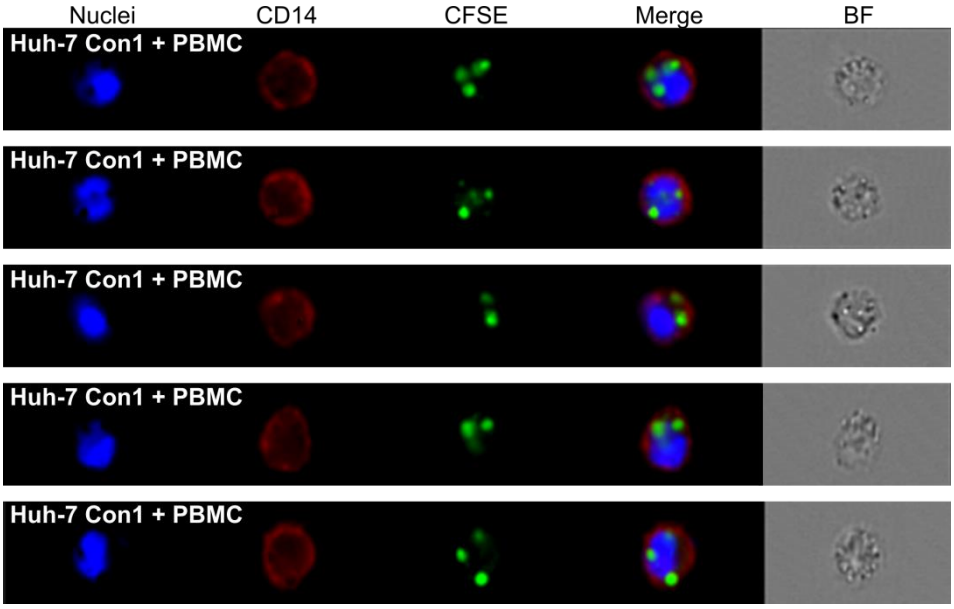

Fig. 4-15 ImageStream analysis of the uptake of HCV SGR cells by monocytes
CFSE-stained Huh-7 Con1 cells were co-cultured with PBMCs overnight. Monocytes were stained by CD14 antibody. Uptake of Huh-7 Con1 cells by monocytes is depicted by ImageStream analysis. Each line represents an independent cellular event.

4.2.6 HCV SGR cells with different backgrounds are taken up by monocytes

To quantify the uptake of Huh-7 Con1 cells by monocytes two parameters were measured by flow cytometry. On the one hand the percentage of CFSE positive monocytes was determined, on the other hand the CFSE MFI of the whole monocyte population was measured. Analysis of these data showed that the uptake of Huh-7 Con1 cells by monocytes within PBMCs was significantly higher compared to Huh-7 and cured Con1 cells (Fig. 4-16 A, B). Moreover, to confirm that the uptake not only occurs with this specific Huh-7 Con1 clone, experiments were repeated with another cell line and another HCV genotype (Huh-6 JFH). Again, uptake of Huh-6 JFH cells was significantly increased compared to Huh-6 or cured JFH cells (Fig. 4-16 C, D).

4.2.7 HCV infected cells are taken up by monocytes

Next to HCV SGR cells, the uptake was also tested with truly HCV infected cells. Indeed, also particles from Huh-7.5 cells infected with the JC1 HCV virus were internalized by monocytes. Importantly, this proof of principle demonstrated that the previous observations with HCV SGR cells were not due to artifacts of HCV SGR cells and that the results are comparable to HCV infected cells.

4.2.8 Monocytes do not take up Dengue Virus and Hepatitis A Virus SGR cells

Having observed that different HCV SGR cells and HCV infected cells were taken up by monocytes, Dengue Virus (DV) and Hepatitis A Virus (HAV) SGR cells were analyzed in addition. Neither DV nor HAV SGR cells induced an uptake by monocytes in the co-culture with PBMCs (Fig. 4-17). This indicated that the PBMC activation observed with HCV SGR cells is specific for HCV, as SGR cells from other RNA viruses from the same family (DV) or with the same tropism (HAV) do not trigger PBMC activation.

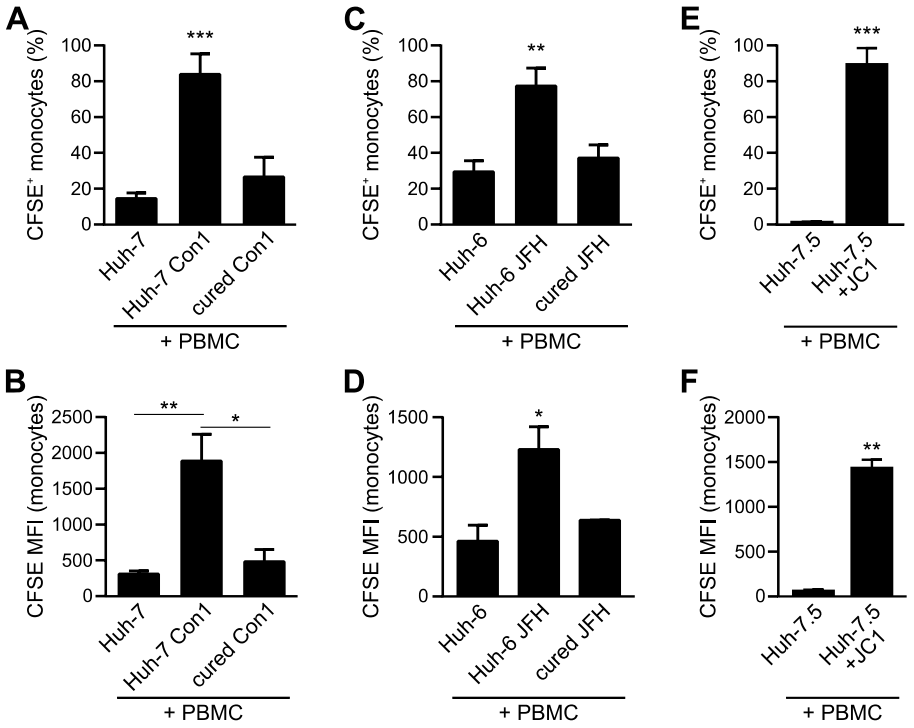

Fig. 4-16 Different HCV SGR cells and HCV infected cells are taken up by monocytes
PBMCs were co-cultured overnight with various CFSE-stained Huh cells and the percentage of CFSE positive monocytes and the CFSE MFI of monocytes were determined by flow cytometry. (A, B) Co-cultures of PBMCs with Huh-7 cells (Con1=HCV genotype 1b) (n=3). (C, D) Co-cultures of PBMCs with Huh-6 cells (JFH=HCV genotype 2a) (n=3). (C) Co-cultures of PBMCs with Huh-7.5 cells or with HCV infected Huh-7.5 cells (Huh-7.5 + JC1) (n=2).

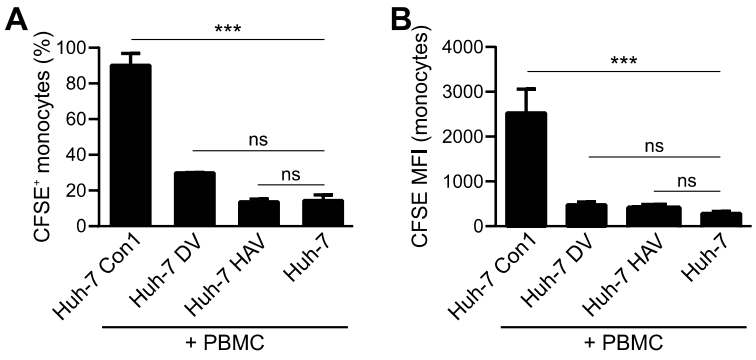

Fig. 4-17 Dengue Virus and Hepatitis A virus SGR cells are not taken up by monocytes
PBMCs were co-cultured overnight with CFSE-stained Huh-7 cells and various Huh-7 SGR cells (Con1 (HCV), Dengue Virus (DV) and Hepatitis A virus (HAV)). Percentage of CFSE positive monocytes (A) and the CFSE MFI of monocytes (B) were determined by flow cytometry (n=3).

4.2.9 Monocyte activation markers CD80 and CD86 are up-regulated in co-culture with HCV SGR cells

Previous results demonstrated that monocytes were taking up particles from HCV SGR cells in co-culture with PBMCs. However, it was not clear if monocytes have an active role in this process or if monocytes only clear remnants of HCV SGR cells. To investigate if monocytes were activated in the co-culture, monocytes were stained for their activation markers CD80 and CD86 and analyzed by flow cytometry. Strikingly, most of the monocytes showed up-regulation of CD80 and CD86 in co-culture of Huh-7 Con1 cells with PBMCs, but not with Huh-7 cells (Fig. 4-18), implying that monocytes play an active role in the anti-viral response against HCV SGR cells.

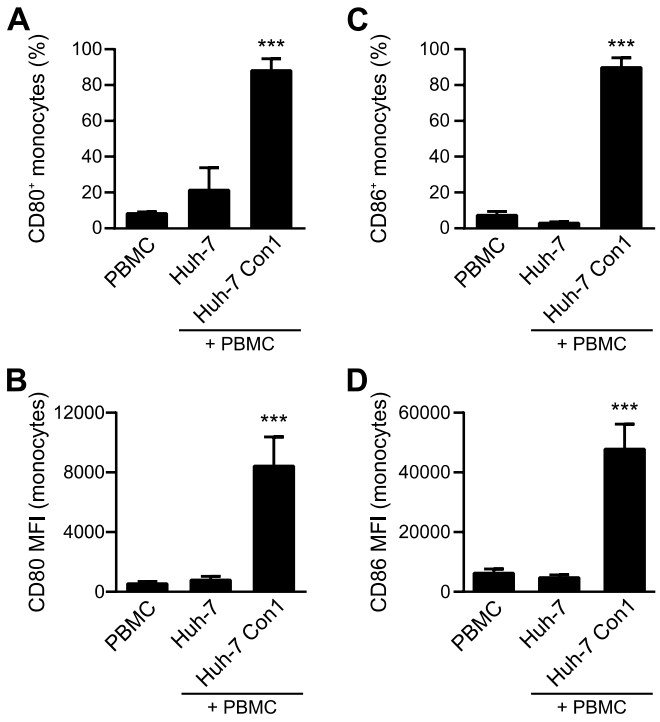

Fig. 4-18 Monocytes are activated in co-cultures of HCV SGR cells with PBMCs
Huh-7 or Huh-7 Con1 cells were co-cultured with PBMCs overnight. Cells were harvested, stained with CD14 antibody to gate on monocytes and with antibodies against the activation markers CD80 (A, B) and CD86 (C, D). CD80 and CD86 positive cells and the MFI of monocytes were analyzed by flow cytometry (n=3).

In summary, in this part it was shown that next to pDCs also NK cells contribute to the anti-viral response by IFNγ secretion. Secondly, also monocytes contribute as monocytes are taking up HCV SGR cells and display up-regulation of activation markers CD80 and CD86. Moreover, several chemokines are secreted by PBMCs in co-culture with HCV SGR cells

and in turn, also HCV SGR cells are triggered by PBMCs to further attract immune cells via secretion of IL-8. Furthermore, the activation of innate immune cells is specific for HCV SGR cells, as DV and HAV SGR were not taken up by monocytes.

4.3 pDCs and NK cells promote uptake of Hepatitis C Virus subgenomic replicon cells by monocytes

4.3.1 Purified monocytes are less efficient in uptake of HCV SGR cells compared to monocytes within PBMCs

As described above, in co-cultures of HCV SGR cells with PBMCs a large proportion of monocytes internalized particles from HCV SGR cells. That raised the question, if this phenotype was caused by monocytes or if additional immune cells were required. Thus, monocytes were MACS-purified and co-cultured with CFSE-stained Huh-7 Con1 cells. The uptake of Huh-7 Con1 cells by purified monocytes was measured by flow cytometry. Only 25.4% of monocytes became CFSE positive in co-culture with Huh-7 Con1 cells, which was significantly more than in co-culture with Huh-7 cells (6%) (Fig. 4-19). However, compared to monocytes within PBMCs (83.6% of monocytes CFSE positive), the percentage was clearly lower. Hence, purified monocytes are able to induce uptake of HCV SGR cells, but the effect is considerably stronger if other immune cells are present.

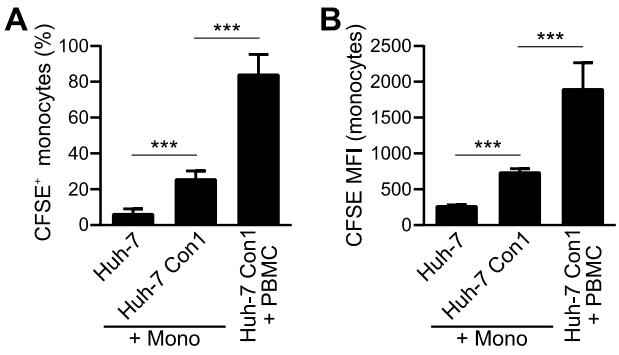

Fig. 4-19 Purified monocytes are less efficient in uptake of HCV SGR cells
Purified monocytes (Mono) or PBMCs were co-cultured with CFSE-stained Huh-7 or Huh-7 Con1 cells overnight. Percentage of CFSE positive monocytes (A) and the CFSE MFI of monocytes (B) were determined by flow cytometry (n=5).

4.3.2 Purified monocytes display only slight up-regulation of CD80 and CD86 in response to HCV SGR cells

In another approach to analyze to what extent purified monocytes are activated by HCV SGR cells, monocyte activation markers CD80 and CD86 were analyzed again. While CD80 and CD86 were strongly up-regulated on monocytes in co-culture of Huh-7 Con1 cells with PBMCs (Fig. 4-18), no significant increase in surface expression of CD80 and CD86 was observed between the co-culture of purified monocytes with Huh-7 or with Huh-7 Con1 cells (Fig. 4-20, note the different scale compared to Fig. 4-18). Nevertheless, a slight increase in co-culture with Huh-7 Con1 cells compared to Huh-7 was observed. In line with the uptake experiments above this again indicated that purified monocytes are activated to some extent, but that other immune cells contribute to monocyte activation.

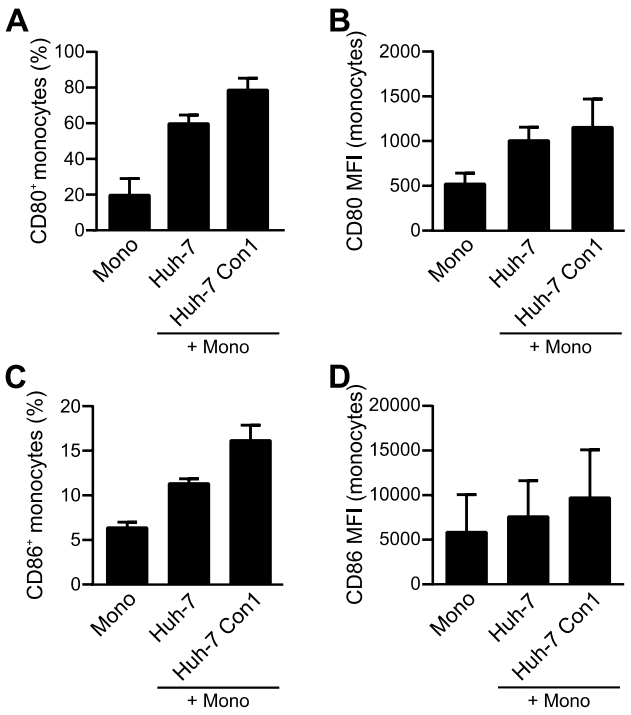

Fig. 4-20 Purified monocytes only slightly up-regulate CD80 and CD86 in response to HCV SGR cells
Huh-7 or Huh-7 Con1 cells were co-cultured with purified monocytes overnight. Cells were harvested, stained with CD14 antibody to gate on monocytes and with antibodies against the activation markers CD80 (A, B) and CD86 (C, D). CD80 and CD86 positive cells and the MFI of monocytes were analyzed by flow cytometry (n=3).

4.3.3 pDCs and NK cells contribute to uptake of HCV SGR cells by monocytes

Since it was observed that activation of purified monocytes in co-culture with HCV SGR cells was lower compared to monocytes within PBMCs, it was speculated that pDCs and NK cells might play a role for the uptake by monocytes as both were shown to secrete IFNα (pDCs, Fig. 4-1) or IFNγ (NK cells, Fig. 4-11) in response to HCV SGR cells. To test this, pDCs and NK cells were depleted from PBMCs and the uptake of Huh-7 Con1 cells by monocytes was analyzed. Interestingly, neither depletion of pDCs nor depletion of NK cells alone reduced the uptake. However, when pDCs and NK cells were both depleted, the uptake significantly dropped to levels similar to the uptake by purified monocytes (Fig. 4-21). These results demonstrated that both, pDCs and NK cells promote the uptake of HCV SGR cells by monocytes.

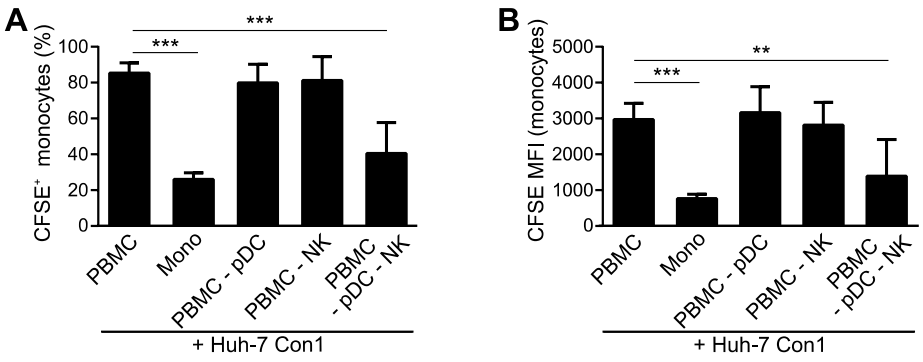

Fig. 4-21 pDCs and NK cells contribute to uptake of HCV SGR cells by monocytes
CFSE-stained Huh-7 Con1 cells were co-cultured overnight with PBMCs, purified monocytes (Mono), PBMCs depleted from pDCs (PBMC -pDC), from NK cells (PBMC -NK) or from pDCs and NK cells (PBMC - pDC -NK). Percentage of CFSE positive monocytes (A) and the CFSE MFI of monocytes (B) were determined by flow cytometry (n=3).

4.3.4 IFNα and IFNγ boost the uptake of HCV SGR cells by monocytes

As it was found that pDCs and NK cells promote the uptake of HCV SGR by monocytes, next it was analyzed if the respective interferons produced by these cells were sufficient to increase the uptake by monocytes. Monocytes were purified and co-cultured with CFSE-stained Huh-7 cells in the presence of IFNα or IFNγ. Concentrations of interferons were derived from the amount measured in the co-culture of HCV SGR cells with PBMCs. Indeed, both interferons significantly boosted the uptake of Huh-7 Con1 cells (Fig. 4-22 A, B). In the presence of IFNα 62.7% of monocytes turned CFSE positive, 40.6% in presence of IFNγ (25.4% without interferon). Interferons had only a minor effect on the uptake of

Huh-7 control cells. In order to analyze if interferons acted on monocytes or on Huh-7 Con1 cells, either monocytes or Huh-7 Con1 cells were pre-incubated with IFNα overnight before setting up the co-culture. However, neither pre-incubation of monocytes with IFNα (Fig. 4-22 C, D) nor of Huh-7 Con1 cells (Fig. 4-22 E, F) was sufficient to significantly boost the uptake. This indicated that IFNα needs to act on monocytes as well as on Huh-7 Con1 cells during the co-culture period to promote uptake.

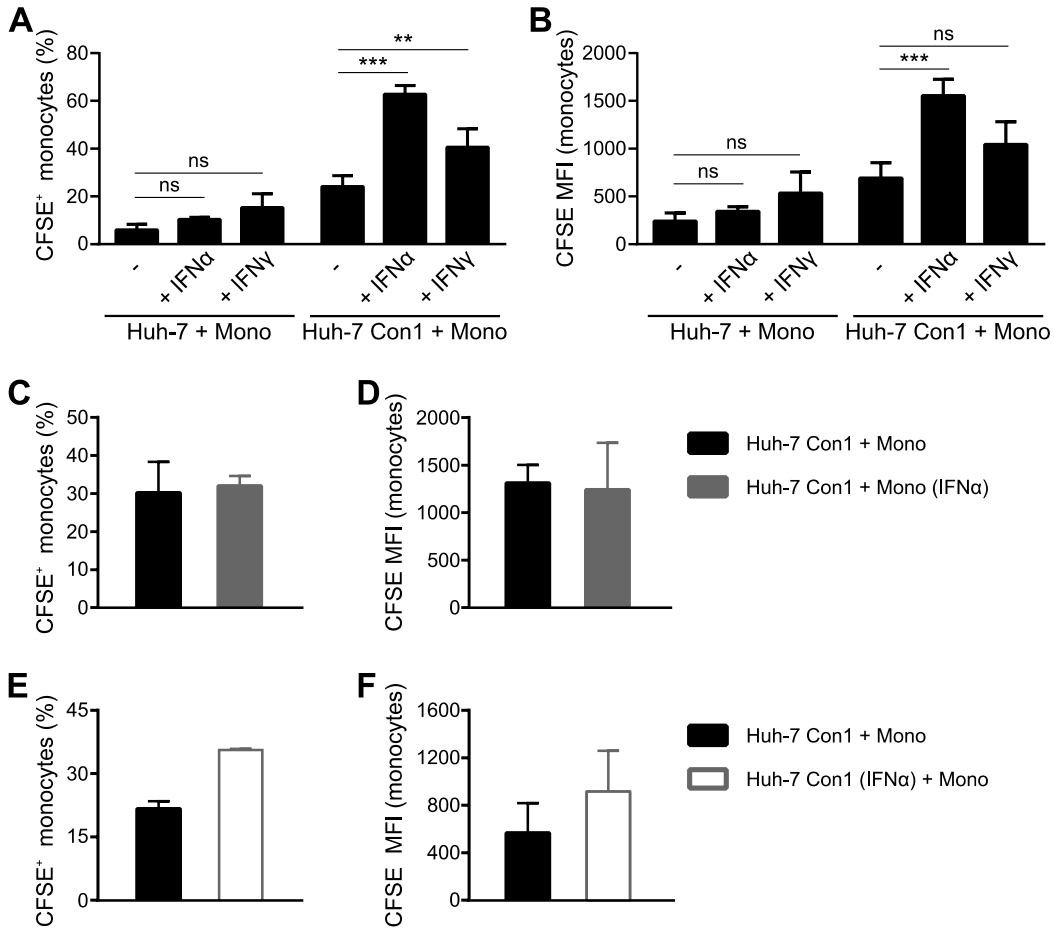

Fig. 4-22 Interferons boost the uptake of HCV SGR cells by monocytes
(A, B) CFSE-stained Huh-7 or Huh-7 Con1 cells were co-cultured overnight with purified monocytes (Mono) in the presence of IFNα (700 pg/ml) or IFNγ (7 ng/ml). (n=4.) (C, D) Purified monocytes were incubated with IFNα overnight, IFNα was removed by washing and monocytes were added to CFSE-stained Huh-7 Con1 cells for overnight co-culture (n=2). (E, F) CFSE-stained Huh-7 Con1 cells were incubated with IFNα overnight, IFNα was removed by washing and purified monocytes were added for overnight co-culture (n=2). Percentage of CFSE positive monocytes (A, C, E) and the CFSE MFI of monocytes (B, D, F) were determined by flow cytometry.

Next to addition of recombinant interferons to purified monocytes, the role of interferons for the uptake by monocytes was also investigated in a time course experiment. CFSE-stained Huh-7 Con1 cells were co-cultured with PBMCs and every 2 hours supernatant

and cells were collected to measure IFNα and IFNγ levels by ELISA and monocyte uptake by flow cytometry (Fig. 4-23). After 6 h around 30% of monocytes were CFSE positive (this was in range with the uptake by purified monocytes), while no IFNα and low amounts of IFNγ were detected. From this time point on monocyte uptake steadily increased and after 14 h 83% of monocytes were CFSE positive. The increase in monocyte uptake correlated with increased levels of IFNγ levels after 8 h and especially after 10 h. In this time range also IFNα was detected. To sum up, monocyte uptake began before secretion of interferons, thus confirming previous results in which purified monocytes were also able to take up HCV SGR cells (Fig. 4-19). However, as soon as interferons were released the uptake was significantly increased, again indicating that interferons boost the uptake by monocytes.

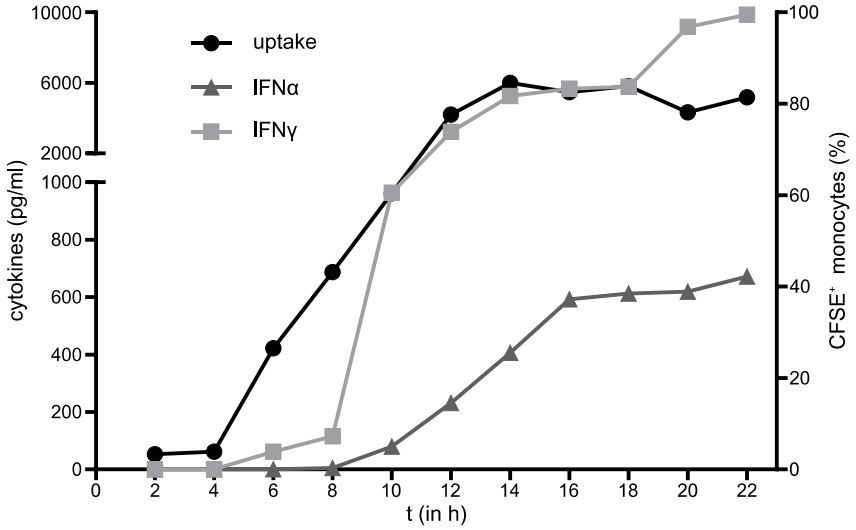

Fig. 4-23 Time course analysis of monocyte uptake and interferon secretion
CFSE-stained Huh-7 Con1 cells were co-cultured with PBMCs. After every 2 h supernatants and cells were harvested and IFNα and IFNγ levels were measured by ELISA. Monocyte uptake was analyzed by flow cytometry (n=1).

4.3.5 Monocytes are required for secretion of IFNγ by NK cells

While previous experiments revealed that pDCs and NK cells influence the uptake of HCV SGR cells by monocytes, it has also been published that monocytes influence NK cells in the context of an anti-viral response against HCV[82]. Hence, monocytes were depleted from PBMCs before co-culture with Huh-7 Con1 cells and interferon secretion was analyzed. IFNα production by pDCs was not affected by monocyte depletion (Fig. 4-24 A). In

contrast, IFNγ secretion by NK cells was almost completely lost (Fig. 4-24 B). Hence, it was confirmed that monocytes influence the NK cell response in co-culture with HCV SGR cells. Moreover, it became clear that not only pDCs and NK cells act on monocytes, but that also monocytes have an effect on NK cells. In addition, this explained why purified NK cells did not respond with IFNγ secretion in co-culture with HCV SGR cells.

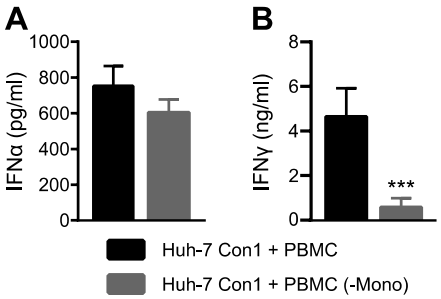

Fig. 4-24 IFNγ secretion by NK cells is dependent on monocytes
Huh-7 Con1 cells were co-cultured with PBMCs or with PBMC depleted from monocytes (-Mono) overnight. Supernatants were collected and measured for IFNα (A) and IFNγ (B) level by ELISA (n=3).

In summary, in this part it was shown that purified monocytes are able to respond to HCV SGR cells. However, their activation is significantly increased when pDCs or NK cells are present and secrete their respective interferons. Moreover, monocytes in turn also influence the response of NK cells, as IFNγ secretion by NK cells is dependent on monocytes. The data indicated that multiple innate immune cells interact with each other in the response against HCV SGR cells.

4.4 Interaction of innate immune cells leads to apoptosis of HCV SGR cells

4.4.1 HCV SGR cells are killed by PBMCs

Following the detailed analysis of the uptake of HCV SGR cells by monocytes and how pDCs and NK cells contribute to this, it was analyzed why the small particles visualized in ImageStream analysis (Fig. 4-14, Fig. 4-15) occur. These particles and the observation that the number of HCV SGR cells decreased during co-culture with PBMCs (Fig. 4-14 C) led to the hypothesis that HCV SGR cells might be killed by PBMCs. To test this

hypothesis an LDH release assays was performed. Huh-7 control cells were not killed, but around 35% of Huh-7 Con1 cells were killed in co-culture with PBMCs (Fig. 4-25 A). In order to analyze if Huh-7 Con1 cells underwent apoptosis, cleavage of PARP was analyzed by Western Blot, as cleaved PARP determines the final irreversible step of apoptosis. Cleaved PARP was only detected in the co-culture of Huh-7 Con1 cells with PBMCs (Fig. 4-25 B), indicating that PBMCs drive HCV SGR cells into apoptosis. Thus, the particles detected within monocytes in previous experiments were apoptotic vesicles from HCV SGR cells.

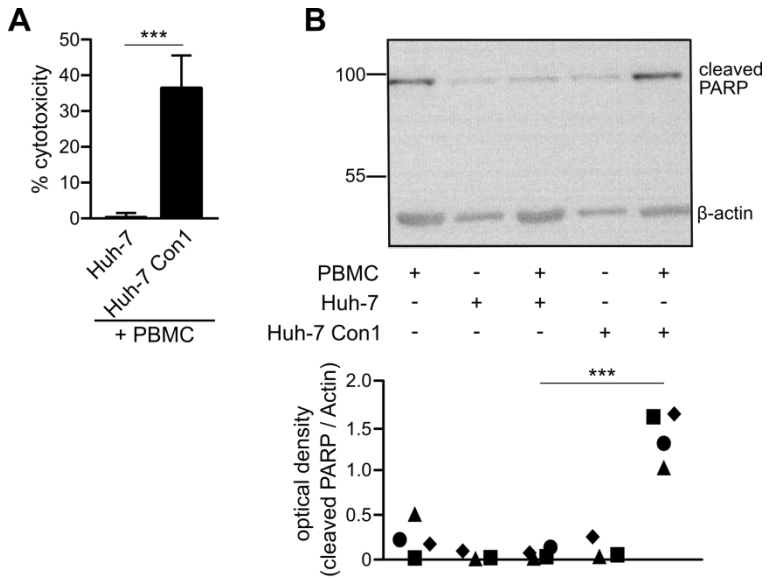

Fig. 4-25 HCV SGR cells are killed by PBMCs
Huh-7 or Huh-7 Con1 cells were co-cultured with PBMCs overnight. Supernatants were collected and LDH release was measured (A) (n=4). Cells were lysed and analyzed for cleavage of PARP by Western Blot (B). In the upper panel in (B) one representative experiment is depicted, the lower panel shows quantification of n=3-4.

4.4.2 Caspase inhibition blocks killing and uptake of HCV SGR cells

PARP cleavage already indicated that HCV SGR cells are driven into apoptosis by PBMCs. This was further analyzed by blocking of caspases, as successive caspase activation leads to apoptosis of target cells. When all caspases were inhibited by a pan-caspase inhibitor, significantly less Huh-7 Con1 cells were killed by PBMCs and, accordingly, less monocytes became CFSE positive (Fig. 4-26). To specifically block a caspase in the target cell, a caspase-8 inhibitor was used. Although not as efficient as the pan-caspase block, killing and uptake of Huh-7 Con1 cells was still significantly decreased.

Blocking caspase-1 was used as control, as caspase-1 is not involved in apoptosis, but plays a role in inflammasome activation. Indeed, caspase-1 inhibition had no effect on killing and uptake of HCV SGR cells by PBMCs. Taken together the data demonstrated that PBMCs induce apoptosis in HCV SGR cells.

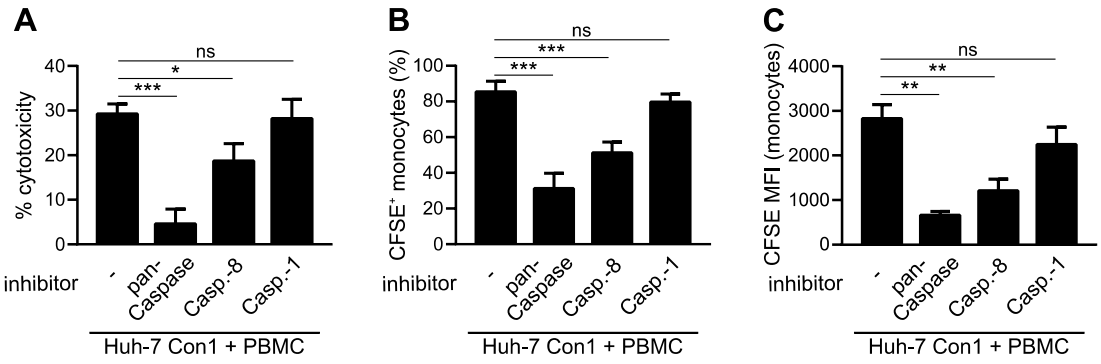

Fig. 4-26 Caspase inhibition blocks killing and uptake of HCV SGR cells
Huh-7 Con1 cells were co-cultured with PBMCs in presence of pan-caspase inhibitor Z-VAD-FMK (20 µM), caspase-8 inhibitor Z-IETD-FMK (20 µM) or caspase-1 inhibitor Z-YVAD-FMK (20 µM). After overnight incubation LDH release was measured in the supernatant (A), percentage of CFSE positive monocytes (B) and the CFSE MFI of monocytes (C) were determined by flow cytometry.

4.4.3 Interaction of innate immune cells is required for killing of HCV SGR cells

Having observed that HCV SGR cells are driven into apoptosis by PBMCs, the question raised which cells within PBMCs trigger apoptosis of HCV SGR cells. Previous results showed that NK cells, pDCs and monocytes were activated in co-culture with HCV SGR cells. Thus, these cells were purified and co-cultured with Huh-7 Con1 cells. LDH release assays were performed to detect apoptosis. However, neither purified NK cells, nor pDCs or monocytes were able to efficiently kill Huh-7 Con1 cells. In contrast, recombination of these cell types led to killing of Huh-7 Con1 cells, although not as many cells were killed as by complete PBMCs (14.1% vs 34.1%, Fig. 4-27 A). The lower percentage of killed Huh-7 Con1 cells might be due to impaired functions of immune cells as a consequence of the purification process. Nevertheless, the experiment demonstrated that interaction of different innate immune cells is necessary and sufficient for killing of HCV SGR cells.

To confirm that multiple innate immune cells contribute to killing of HCV SGR cells, NK cells, pDCs or monocytes were depleted from PBMCs and killing activity was measured by an LDH release assay. Independent of which cell type was depleted, the percentage of

killed Huh-7 Con1 cells slightly decreased (although not significantly) (Fig. 4-27 B), indicating that each cell type plays a role in induction of apoptosis of HCV SGR cells. In line with this, depletion of both, NK cells and pDCs significantly reduced cytotoxicity, highlighting the need for combined activation and interplay of different innate immune cells to kill HCV SGR cells.

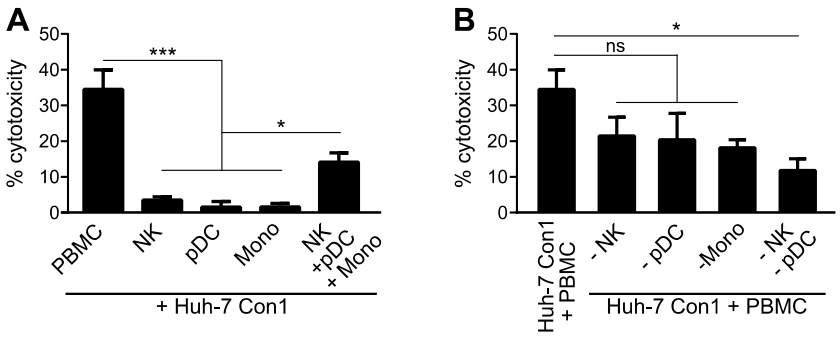

Fig. 4-27 Interaction of innate immune cells is required for killing of HCV SGR cells
(A) Huh-7 Con1 cells were co-cultured overnight with PBMCs, purified NK cells (NK), purified pDCs (pDC), purified monocytes (Mono) or a recombination of NK cells, pDCs and monocytes (NK +pDC +Mono). Supernatants were collected and LDH release was measured (n=3). (B) Huh-7 Con1 cells were co-cultured overnight with PBMCs, PBMCs depleted from from NK cells (-NK), from pDCs (-pDC), from monocytes (-Mono) or from pDCs and NK cells (-NK -pDC). Supernatants were collected and LDH release was measured (n=3).

4.4.4 NK cells display surface expression of CD69, CD25 and TRAIL in co-culture of HCV SGR cells with PBMCs

As the above described experiments showed that interaction of innate immune cells is required to cause apoptosis in HCV SGR cells, next the mechanisms of triggering apoptosis were investigated. NK cells were more closely examined as they are known to be able to kill virus infected cells. Thus, NK cells were analyzed for expression of activation markers in the co-culture of Huh-7 Con1 cells with PBMCs by flow cytometry. A strong up-regulation of the activation markers CD69 and CD25 on NK cells was observed in co-culture of Huh-7 Con1 cells and PBMCs (Fig. 4-28 A, B). In combination with the previously described IFNγ secretion by NK cells (Fig. 4-11), this observation underlined the hypothesis that NK cells are able to induce apoptosis in HCV SGR cells. Indeed, up-regulation of TRAIL was found on NK cells which is known to induce apoptosis in target cells (Fig. 4-28 C). Interestingly, only a subpopulation of NK cells expressed TRAIL (Fig. 4-28 right plot), suggesting that a specific subtype of NK cells is responsible for apoptosis induction in HCV SGR cells.

4.4.5 Blocking TRAIL reduces killing and uptake of HCV SGR cells

In order to analyze if the observed TRAIL expression on NK cells causes apoptosis in HCV SGR cells, Huh-7 Con1 cells were co-cultured with PBMCs in presence of a TRAIL blocking antibody. Strikingly, blocking TRAIL significantly decreased cytotoxicity of PBMCs against Huh-7 Con1 cells (Fig. 4-29 A). In consequence, also the uptake by monocytes of CFSE labeled Huh-7 Con1 cells was reduced (Fig. 4-29 B, C). Addition of an IgG control antibody had no influence on killing and uptake of Huh-7 Con1 cells. Thus, it was proven that TRAIL expression on NK cells drives apoptosis of HCV SGR cells.

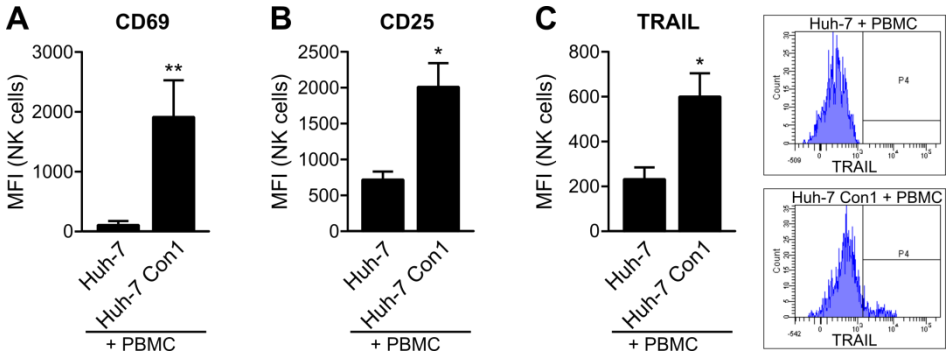

Fig. 4-28 CD69, CD25 and TRAIL are expressed on NK cells in co-culture with HCV SGR cells
Huh-7 or Huh-7 Con1 cells were co-cultured with PBMCs overnight. Cells were harvested, stained with CD3 and CD56 antibody to gate on NK cells ($CD3^-$ $CD56^+$) and with antibodies against the activation markers CD69 (A) and CD25 (B) and against the apoptosis inducing TRAIL (C). MFI of NK cells for each surface protein was analyzed by flow cytometry (n=4). Right plots in (C) depict one representative experiment, in which TRAIL expression on NK cells was measured in co-culture of PBMCs with Huh-7 cells (top) or with Huh-7 Con1 cells (bottom).

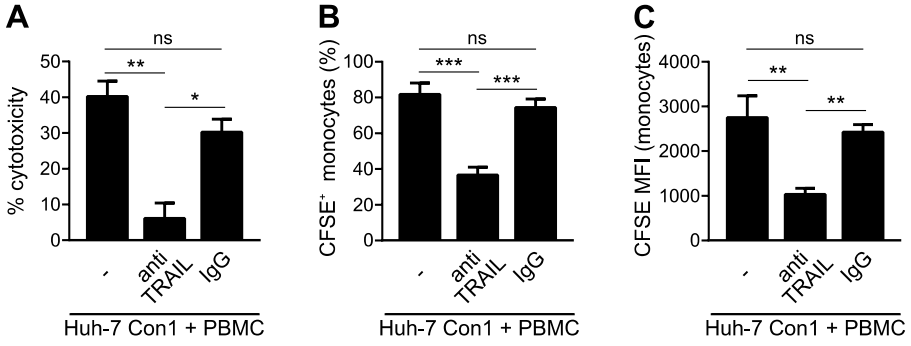

Fig. 4-29 Blocking TRAIL inhibits killing and uptake of HCV SGR cells
Huh-7 Con1 cells were co-cultured with PBMCs in presence of a TRAIL blocking antibody or an IgG control antibody (each 10 µg/ml). After overnight incubation LDH release was measured in the supernatant (A), percentage of CFSE positive monocytes (B) and the CFSE MFI of monocytes (C) were determined by flow cytometry (n=4).

4.4.6 TRAIL receptor expression is similar in HCV SGR cells and control cells

Since HCV SGR cells were driven into apoptosis by TRAIL expression on NK cells, it was investigated if susceptibility of HCV SGR cells is due to overexpression of TRAIL receptors. To this end, RNA from Huh-7 and Huh-7 Con1 cells was extracted and analyzed for TRAIL receptor expression by qPCR. All known TRAIL receptors were analyzed, the pro-apoptotic receptors DR4 (death receptor 4) and DR5 as well as the anti-apoptotic receptors DcR1 (decoy receptor 1) and DcR2. However, none of the TRAIL receptors were differentially expressed (mRNA) between Huh-7 and Huh-7 Con1 cells (Fig. 4-30 A-C). DcR1 was not expressed (data not shown).

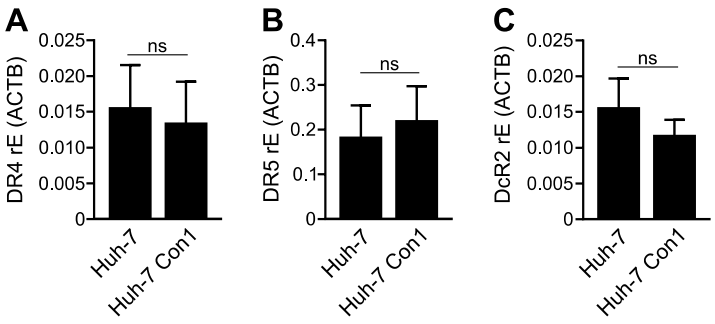

Fig. 4-30 TRAIL receptor expression is comparable in Huh-7 and Huh-7 Con1 cells
Huh-7 and Huh-7 Con1 cells were analyzed for expression of pro-apoptotic TRAIL receptors DR4 (A) and DR5 (B) and for anti-apoptotic TRAIL receptor DcR2 (C) by qPCR.

4.4.7 Activated PBMCs target HCV SGR cells, but not naïve Huh-7 cells

Following the observation of similar TRAIL receptor expression in Huh-7 and Huh-7 Con1 cells, it was examined if PBMCs pre-activated by HCV SGR cells could also target Huh-7 control cells. This was investigated in a triple co-culture of Huh-7, Huh-7 Con1 cells and PBMCs. Before analyzing if also Huh-7 cells are killed in this triple co-culture, it had to be proven that PBMCs are activated in this setup. Thus, the triple co-culture was performed and different parameters for PBMC activation were measured. IFNα secretion by pDCs, IFNγ secretion by NK cells and NK cell activation markers CD25 and CD69 were detected in the triple co-culture (Fig. 4-31). Compared to the co-culture of Huh-7 Con1 cells with PBMCs the signals were lower for each readout, which can be explained by the lower numbers of Huh-7 Con1 cells. In the triple co-culture only half as many Huh-7 Con1 cells are present compared to the co-culture of Huh-7 Con1 cells with PBMCs.

Having shown that PBMCs are activated in the triple co-culture, it was analyzed if also Huh-7 cells are targeted by PBMCs that are stimulated by HCV SGR cells. Therefore, Huh-7 Con1 cells were stained with CFSE, Huh-7 cells were stained with CytoRED and the triple co-culture with PBMCs was set up.

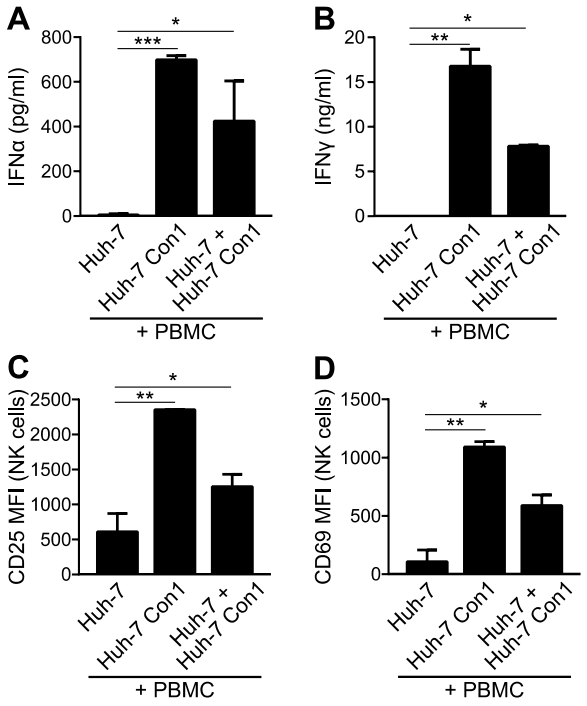

Fig. 4-31 Innate immune cells are activated in triple co-culture of Huh-7, Huh-7 Con1 and PBMCs
(A-D) PBMCs were co-cultured overnight with Huh-7, Huh-7 Con1 or with Huh-7 and Huh-7 Con1 cells together. Supernatants were harvested and analyzed by ELISA for IFNα (A) and IFNγ (B). Cells were harvested and CD25 (C) and CD69 (D) expression on NK cells was analyzed by flow cytometry (n=2).

To analyze if only Huh-7 Con1 or also Huh-7 cells were killed, it was measured by flow cytometry whether monocytes turned CFSE positive or CytoRED positive. As a result monocytes became CFSE positive, indicating that Huh-7 Con1 cells were killed. In contrast, monocytes did not become CytoRED positive (Fig. 4-32), showing that naïve Huh-7 control cells were not driven into apoptosis by PBMCs that were activated in the same culture by presence of HCV SGR cells. To exclude an influence of the dyes, staining of Huh-7 cells was performed vice versa. Thus, Huh-7 cells were stained with CFSE and Huh-7 Con1 cells with CytoRED. Again, only Huh-7 Con1 but not Huh-7 cells were killed by PBMCs, as monocytes only became CytoRED positive (Fig. 4-33). Taken together, these results demonstrated that PBMCs specifically kill HCV SGR cells, but do not target uninfected control cells.

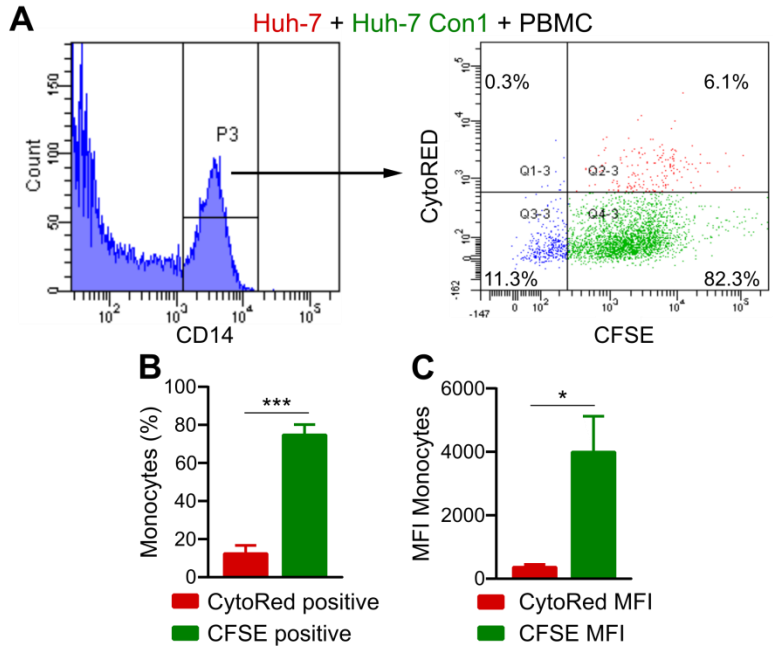

Fig. 4-32 Activated PBMCs target HCV SGR cells, but not naïve Huh-7 cells
CytoRED-stained Huh-7 cells were co-cultured with CFSE-stained Huh-7 Con1 cells and PBMCs overnight. The percentage of CytoRED and CFSE positive monocytes (B) as well as the CytoRED and CFSE MFI (C) of monocytes were measured (n=3). The gating strategy is depicted in (A).

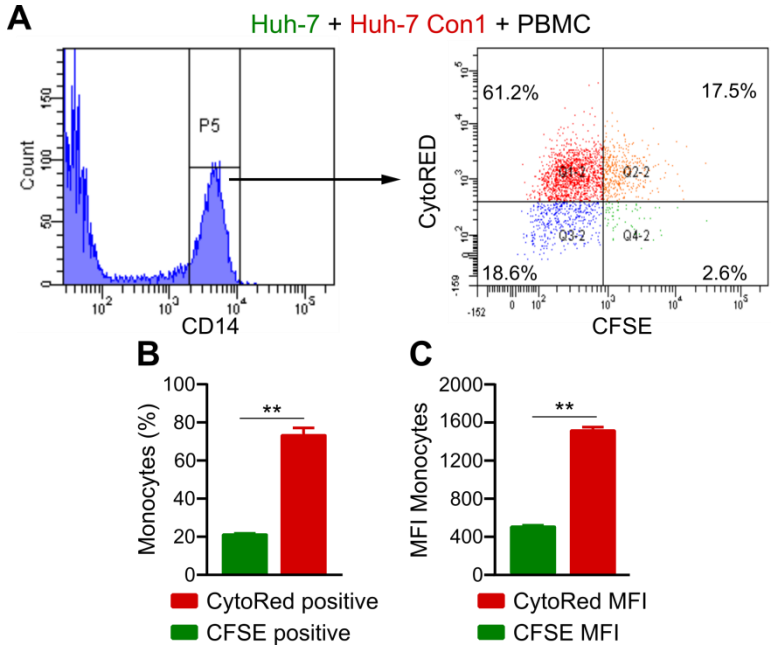

Fig. 4-33 Activated PBMCs target HCV SGR cells, but not naïve Huh-7 cells
CFSE-stained Huh-7 cells were co-cultured with CytoRED-stained Huh-7 Con1 cells and PBMCs overnight. The percentage of CFSE and CytoRED positive monocytes (B) as well as the CFSE and CytoRED MFI (C) of monocytes were measured (n=3). The gating strategy is depicted in (A).

4.4.8 NK cells adhere stronger to HCV SGR cells compared to Huh-7 cells

As PBMCs in the triple co-culture with Huh-7 and Huh-7 Con1 cells only targeted the latter ones, it was speculated that up-regulation of surface ligands on HCV SGR cells could be recognized by PBMCs. Thus, the adherence of PBMCs to Huh-7 and Huh-7 Con1 cells were measured by flow cytometry. After overnight co-culture, non-adherent cells were washed off and collected. Adherent cells were detached by trypsin treatment and separately collected from washed off cells. The percentages of monocytes and NK cells in these two fractions were then analyzed by flow cytometry. For monocytes no difference in adherence to Huh-7 or Huh-7 Con1 cells was observed, as monocytes per se are strongly adherent cells (Fig. 4-34 A). Yet, NK cells displayed stronger adherence to Huh-7 Con1 cells (Fig. 4-34 B). While in the co-culture with Huh-7 cells more NK cells were found in the washed off cells, in co-culture with Huh-7 Con1 cells more NK cells were present in the fraction detached by trypsin. Hence, this result supported the hypothesis that ligands on HCV SGR cells might be recognized by PBMCs.

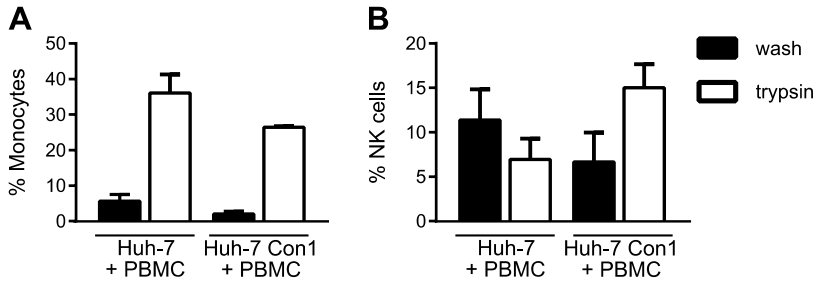

Fig. 4-34 Adherence of monocytes and NK cells to Huh-7 cells and Huh-7 Con1 cells
Huh-7 or Huh-7 Con1 cells were co-cultured with PBMCs overnight. Non-adherent cells were washed off and adherent cells were detached by trypsin treatment. Both fractions were stained with CD56 antibody to gate on NK cells and with CD14 antibodies to gate on monocytes. Percentage of monocytes (A) and NK cells (B) in both fractions were determined by flow cytometry (n=3).

4.4.9 Analysis of the surface proteome of HCV SGR cells

To study the hypothesis of differential expression of surface proteins between HCV SGR cells and Huh-7 control cells as a trigger for PBMC activation, the surface proteome of both cells was analyzed. In brief, surface proteins were biotinylated, purified by streptavidin beads and then analyzed by mass spectrometry. Four proteins were found to be significantly up-regulated on Huh-7 Con1 cells compared to Huh-7 cells: SQRDL (sulfide quinone reductase-like protein), ACTA1 (alpha-actin-1), RPS6KA3 (ribosomal

protein S6 kinase alpha-3). SMPDL3B (sphingomyelin phosphodiesterase acid like 3B) (Fig. 4-35 A). Despite multiple efforts to eliminate intracellular proteins, it was impossible to completely remove all. Thus, only one of these four proteins in fact is a typical surface protein, SMPDL3B. However, this protein was shown to be a negative regulator of innate immunity[119] and was not further considered to be responsible for activation of innate immune cells in co-culture with HCV SGR cells.

Nevertheless, the surface proteome revealed that CD38 and CD44 were also up-regulated on Huh-7 Con1 cells (although not significant). Both proteins were also found in unpublished microarray data (AG Lohmann) to be higher expressed in HCV SGR cells. Hence, the expression of CD38 and CD44 was analyzed by flow cytometry. Indeed, surface expression of both proteins was higher on Huh-7 Con1 cells compared to Huh-7 cells (Fig. 4-35 B, C). Especially CD38 was of high interest as it is a ligand for CD31 which is highly expressed on monocytes. However, neither blocking of CD38 or of CD31 by antibodies, nor siRNA mediated knockdown of CD38 had an effect on activation of PBMCs. Similarly, also CD44 had no influence (data not shown). In summary, the hypothesis that PBMCs recognize HCV SGR cells by expression of specific surface proteins could not be substantiated by additional data.

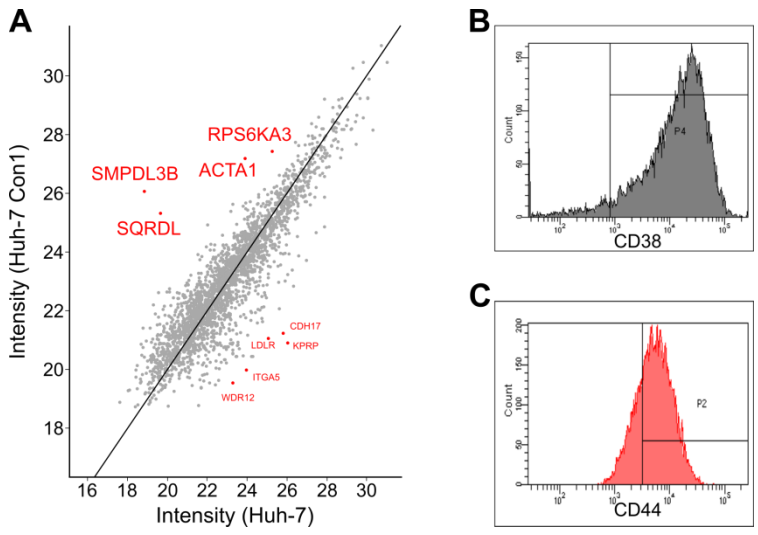

Fig. 4-35 Analysis of the surface proteome of HCV SGR cells
(A) Surface proteins of Huh-7 and Huh-7 Con1 cells were biotinylated, cells were lysed, biotinylated proteins were captured by streptavidin beads and analyzed by mass spectrometry. Red dots depict significantly differentially expressed proteins (n=1). (B, C) Huh-7 Con1 cells were stained with CD38 (B) or CD44 (C) antibodies and analyzed by flow cytometry. Gates were set with Huh-7 cells, one representative experiment of n=2 is shown.

4.4.10 HCV SGR cells are sensitized towards TRAIL signaling

Since surface proteome analysis could not explain why PBMCs (activated by HCV SGR cells) specifically kill HCV SGR cells but not Huh-7 cells, another hypothesis was tested. Previous results indicated that NK cells kill HCV SGR cells by TRAIL expression (Fig. 4-28, Fig. 4-29), but TRAIL receptor expression was similar in Huh-7 and in Huh-7 Con1 cells (Fig. 4-30). Thus, it was analyzed if HCV SGR cells per se are more sensitive towards TRAIL induced apoptosis. Therefore, Huh-7 Con1 cells were treated with recombinant TRAIL and killing of cells was investigated. Strikingly, only Huh-7 Con1 cells, but not Huh-7 or cured Con1 cells were killed by recombinant TRAIL (Fig. 4-36 A). Interestingly, similar to the co-culture with PBMCs around 30% of cells were killed. In contrast to TRAIL, TNFα (same protein family as TRAIL) did not induce cell death (Fig. 4-36 B), suggesting that the effect is specific for TRAIL. To prove that recombinant TRAIL induced apoptosis in Huh-7 Con1 cells, PARP cleavage was analyzed by Western Blot. Indeed, cleaved PARP was detected only detected in Huh-7 Con1 cells treated with TRAIL (Fig. 4-36 C, D). Finally, the sensitivity of HCV SGR cells towards TRAIL explains why activated PBMCs specifically kill HCV SGR cells but not Huh-7 cells.

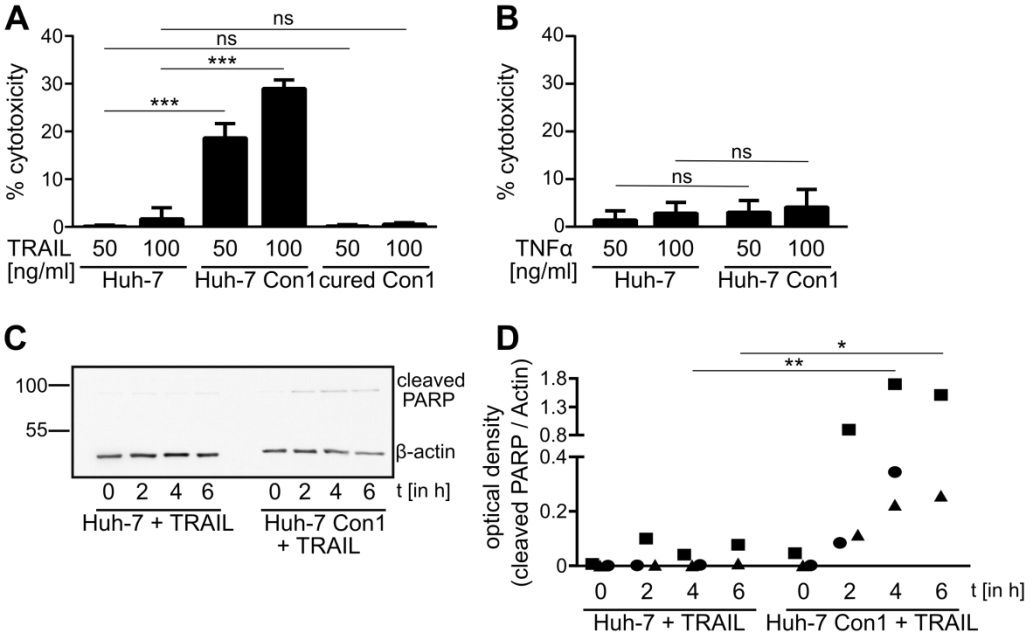

Fig. 4-36 TRAIL induces apoptosis of HCV SGR cells
(A, B) Huh-7 cells were incubated with recombinant TRAIL (A) or TNFα (B) at the indicated concentrations overnight. Cell free supernatants were collected and measured for LDH activity (n=3). (C, D) Huh-7 cells were incubated with recombinant TRAIL (100 ng/ml) for the indicated time points and analyzed for cleavage of PARP by Western Blot. One representative blot is shown in (C). Quantification of n=2-3 is shown in (D).

In summary, the first parts of this work showed that multiple innate immune cells (pDCs, NK cells, monocytes) contribute to the response against HCV SGR cells. Eventually, in this last part it was demonstrated that the combined activation and interplay of these innate immune cells leads to killing and clearance of HCV SGR cells. As a mechanism underlying killing, TRAIL expression on NK cells was found to drive apoptosis in HCV SGR cells. Importantly, control cells were not targeted as only HCV SGR cells are sensitive towards TRAIL.

5 Discussion

Previous studies had shown that ISGs are up-regulated in livers of patients with chronic HCV infection. Despite different mechanisms of HCV to interfere with intracellular immune responses, somehow interferons must be produced that account for the up-regulation of ISGs. Recently, it was discovered that pDCs are able to respond to HCV infected cells or to SGR cells by production of IFNα[59]. In the present study the innate immune response against HCV was investigated in detail by co-culturing HCV SGR cells with PBMCs. The results demonstrate how the interaction of various innate immune cells enables them to elicit an efficient, rapid and specific response against HCV infected cells. While PBMCs were able to kill and clear HCV SGR cells (Fig. 4-25, Fig. 4-14), purified subsets of innate immune cells were not able to kill HCV SGR cells (Fig. 4-27). Effector mechanisms of immune cells exerted in response to HCV SGR cells within complete PBMCS did not occur (or were weaker) with purified immune cells. For example, NK cells within PBMCs secreted IFNγ (Fig. 4-11 A, B), whereas purified NK cells failed to do so (Fig. 4-11 C). On the other hand, monocytes displayed strong up-regulation of activation markers CD80 and CD86 within PBMCs (Fig. 4-18), but purified monocytes only showed a minor non-significant up-regulation (Fig. 4-20). The contribution of individual innate immune cells and their interplay in the response against HCV will be discussed below.

5.1 pDC activation in response to HCV SGR cells

As earlier reports indicated that pDCs respond to HCV infected cells[58,59], in a first step to establish the experimental setup of the co-culture of HCV SGR cells and PBMCs, IFNα secretion was analyzed. Indeed, these earlier studies could be confirmed, since IFNα was detected in the co-culture of Huh-7 Con1 cells and PBMCs and absent after pDC depletion (Fig. 4-1 A, B). In contrast, cured Con1 cells did not trigger pDCs. Importantly, also purified pDCs responded with IFNα secretion in co-culture with Huh-7 Con1 cells (Fig. 4-1 C), indicating that pDCs directly recognize HCV replicating cells.

5.1.1 HCV RNA as stimulus for immune cell activation

Previous reports suggested a role of viral RNA in pDC activation[59,118], as IRS661 (a TLR7-inhibitory sequence) significantly decreased IFNα production. Furthermore, fluorescence in

situ hybridization (FISH) experiments showed that 90% of pDCs that carried viral RNA were positive for IFNα[63]. This implied that viral RNA is recognized by TLR7 in pDCs. Indeed, it was reported that HCV RNA can be detected by TLR7[120].

Here it was further investigated if in fact viral RNA is responsible for pDC activation. The amount of HCV RNA in the supernatant of the co-culture correlated with the levels of IFNα secretion by pDCs (Fig. 4-3). Moreover, inhibition of endosomal TLRs by Bafilomycin and Chloroquine completely abrogated IFNα production (Fig. 4-4). This underlines that viral RNA plays a role in pDC activation. To mechanistically analyze the contribution of viral RNA recognition in more detail, the co-culture system was established with murine cells. To this end, BMDM from WT, TLR7 KO mice and mutant Unc93b1 mice (which lack endosomal trafficking of TLRs) were co-cultured with Hep56D SGR cells. While BMDM from TLR7 KO mice displayed reduced cytokine secretion, BMDM from mutant Unc93b1 mice were not stimulated in co-culture with murine SGR cells (Fig. 4-5). These results showed that recognition of viral RNA by TLR7 contributes to stimulation of BMDM. However, as BMDM from TLR7 KO mice still produced low amounts of IL-6, also other mechanisms of stimulation exist (potentially via recognition of dsRNA by TLR3). Experiments with mutant Unc93b1 cells proved that other endosomal TLRs also recognize viral RNA, as BMDM from those mice did not respond.

Next to TLR7, other TLRs recognizing nucleic acids are present in endosomes (TLR 3, 8, 9 and TLR13 in addition in mice)[121]. Since TLR8 in mice is not functional[122], TLR9 recognizes DNA[123], and TLR13 recognizes bacterial RNA[124], TLR3 might sense HCV RNA. TLR3 recognizes dsRNA[38] and dsRNA occurs as a replication intermediate. Therefore, it is possible that a TLR3-dependent activation of BMDMs takes place. Furthermore, positive and negative viral ssRNA are secreted by HCV SGR cells[27] which also indicates that dsRNA could be subjected to immune cells. In fact, the observed contribution of TLR3 in the murine system was also shown to play a role in HCV infected hepatocytes[125]. In that study Huh-7.5 cells were stably transfected with TLR3 (TLR3 is expressed in primary human hepatocytes). After HCV infection these cells were able to sense HCV infection which resulted in NF-κB activation and expression of several cytokines and chemokines. Also monocyte derived macrophages were shown to recognize HCV dsRNA via TLR3[126,127]. Transfection of HCV dsRNA into monocyte derived macrophages led to expression of type I interferons and increased levels of TLR3. Next to TLR3, macrophages were also shown to detect HCV RNA via TLR7 and TLR8[128].

In summary, the results in this study are in line with other reports and provide further evidence that viral RNA is triggering pDC activation via recognition by TLR7. Additionally, the murine model suggested a role of TLR3 in recognition of HCV RNA, which was confirmed in human cells by further reports.

5.1.2 Transport of viral RNA: exosomes versus cell-cell contact

As discussed above, activation of innate immune cells depends on recognition of viral RNA by TLRs. Logically, the question arises how viral RNA accesses innate immune cells. In an earlier study it was reported that viral RNA is secreted from infected cells via exosomes[63]. Other reports however suggested that cell-cell contacts between infected cells and immune cells are required for activation[59].

In this work it was addressed in how far these suggested transport routes account for trafficking of HCV RNA into immune cells. Hence, exosome release inhibitors GW4869 and Spiroepoxide were used to analyze the role of exosomes. While GW4869 reduced pDC activation in co-culture with Huh-7 Con1 cells (Fig. 4-6 A), Spiroepoxide had no effect (Fig. 4-6 C). GW4869 and Spiroepoxide are structurally unrelated, but both act via inhibition of neutral sphingomyelinases[116,129]. Neutral sphingomyelinases regulate the biosynthesis of the sphingolipid ceramide, which triggers the budding of endosomes into MVB[116]. As both inhibitors act via the same mechanism, it was surprising to obtain varying results with the different inhibitors. In another approach to target exosome release, Rab27a was knocked down, which is linked to exosome release[117]. Knockdown of Rab27a significantly reduced IFNα secretion (Fig. 4-6 D), but was not as potent as GW4869. Due to the varying results with the inhibitors and the siRNA treatment, no clear conclusion on the role of exosomes could be drawn at this stage, but exosome trafficking pathway seem to contribute to pDC activation.

Hence, exosomes were purified from HCV SGR cells and it was analyzed if they trigger IFNα production. Despite high levels of HCV RNA in purified exosomes (144x more compared to the co-culture) no activation of pDCs was observed (Fig. 4-7). In contrast, in a previous study isolated exosomes from HCV SGR cells were able to stimulate purified pDCs[63]. However, here exosomes were incubated with complete PBMCs, while Dreux et al. used purified pDCs. Thereby, all exosomes can be taken up by pDCs, while in the setup with complete PBMCs other cell types might shield the low abundant pDCs from

exosomes. In conclusion, exosomal transport of viral RNA seems to allow activation of pDCs. However, as in the direct co-culture of HCV SGR cells with PBMCs lower amounts of viral RNA were sufficient to trigger pDCs (144x less compared to purified exosomes in this study, over 150x fold less compared to purified exosomes in the study of Dreux et al.[63]), direct cell-cell contacts seem to play a superior role in activation of innate immune cells via transfer of viral RNA. Still, it is possible that the transfer of viral RNA during cell-cell contacts is mediated by exosomes, as exosomes were shown to carry viral RNA (Fig. 4-7). It could be speculated that direct contact between infected cells and pDCs triggers a secretion of exosomes in infected cells. Due to the close contact, exosomes containing viral RNA could be directly subjected to pDCs.

The conclusion that cell-cell contacts are of high importance was supported by further results of this study. In transwell experiments where PBMCs were separated from HCV SGR cells by a membrane (with 1 µm large pores which means exosomes could pass) IFNα was not measured (Fig. 4-8 A). Also in experiments where the co-culture of HCV SGR cells was performed with mechanical shaking to prevent formation of cell-cell contacts IFNα was significantly reduced, while shaking did not impair R848 stimulation of pDCs (Fig. 4-8 B). Moreover, in line with a previous study[118], blocking CD81 and CD9 inhibited IFNα secretion by pDCs (Fig. 4-9). CD81 and CD9 both are part of the tetraspanin protein family and interact with each other[130]. Interestingly, pDCs highly express CD81 and CD9, while Huh-7.5 cells show high CD81 and low CD9 levels[118]. Of note, despite expression of the HCV entry receptor CD81 on pDCs, PBMCs are not permissive for HCV infection[131].

The results in this work and from other groups show that pDC activation is clearly more efficient when cell-cell contacts with HCV SGR cells are possible. On one side this might be due to a direct transfer of viral RNA into pDCs, but it is tempting to speculate that this interaction might increase pDC activation due to additional signals comparable to co-stimulatory molecules in immunological synapses[132]. A similar hypothesis was also formulated by other authors[133]. The above discussed interaction between CD81 and CD9 could be part of an interaction within a potential synapse. Also the observed up-regulation of CD38 and CD44 in HCV SGR cells (Fig. 4-35 B, C) could play a role. Especially, CD38 might be involved as it interacts with CD31[134] and CD31 is highly expressed on monocytes[135]. However, blocking CD31 or CD38 and siRNA mediated knockdown of CD38 in HCV SGR cells had no effect on monocyte uptake in the co-culture of HCV SGR

cells with PBMCs or with purified monocytes (data not shown). Nevertheless, if in fact a synapse with multiple interactions between HCV infected cells and immune cells is formed, eliminating one molecule of interaction might not be sufficient. Thus, formation of synapses should be studied by other methods like microscopy.

5.2 NK cell activation in response to HCV SGR cells

Next to IFNα secretion by pDCs, it was analyzed by a Luminex assay if also other cytokines were produced in the co-culture of HCV SGR cells and PBMCs (Fig. 4-10). Secretion of specific cytokines or chemokines could provide evidence that other innate immune cells contribute to the anti-viral response. Indeed, IFNγ secretion was detected in the Luminex assay, which indicated an activation of NK cells (a role of T cells was excluded as the short overnight co-culture is not sufficient to allow a response of adaptive immune cells from healthy donors). This was further confirmed by ELISA (Fig. 4-11 A, B). But interestingly, purified NK cells did not respond with IFNγ secretion (Fig. 4-11 C), suggesting that NK cells need interaction with other immune cells. Monocyte depletion from PBMCs resulted in nearly complete loss of IFNγ (Fig. 4-24). This demonstrated that IFNγ secretion by NK cells depends on monocytes, which is in line with previous findings[82]. In that study it was shown that monocytes react with inflammasome activation to HCV SGR cells, which leads to IL-18 secretion which in turn drives NK cell activation and IFNγ secretion. Here, up-regulation of IL-18 was also detected in the Luminex assay (Fig. 4-10). Moreover, it has been shown that also pDC derived IFNα has an influence on NK cell activation in an HCV context[102]. In the latter study it was observed that purified NK cells failed to secrete IFNγ and by depletion and cytokine stimulation experiments that pDCs and monocytes drive NK cell activation. Also Kupffer cells, the liver resident macrophages, have been shown to be able to activate NK cells in HCV infected livers[136,137]. These studies demonstrated that Kupffer cells respond to TLR ligands and drive NK cell activation by cytokine secretion (e.g. IL-18). Thereby, the results indicate the relevance of the in vitro experiments in this work and other studies.

5.3 Monocyte activation in response to HCV SGR cells

5.3.1 Chemokine secretion indicates monocyte activation

As discussed before, NK cell activation in the co-culture with HCV SGR cells depends on monocytes. Accordingly, monocytes first have to be activated before they can drive NK cell activation. In the Luminex assay a set of cytokines and chemokines were up-regulated that hinted at a role of monocytes (TNFα, IL-6, IL-8, MCP-1, IP-10, MIP-1α and MIP-1β, Fig. 4-10) as all of them can be secreted by monocytes[138–141]. Expression of these cytokines and chemokines was also confirmed by qPCR (Fig. 4-12). Interestingly, others have observed a similar expression profile of cytokines and chemokines in co-cultures of HCV SGR cells with PBMCs[82]. In that study also expression of TNFα, MCP-1, IP-10 and MIP-1β was detected.

Of particular interest was the secretion of IP-10, as this was described to be expressed in patients with HCV infection. Hepatocytes are a major source of IP-10 during HCV infection in vivo and in vitro[142,143]. The production of IP-10 in the liver leads to the recruitment of T cells, NK cells, and monocytes[144]. It has also been shown that IP-10 is induced primarily through an IFN-independent pathway following PRR signaling in the HCV-infected hepatocyte in vitro and that type I and type III IFNs produced by non-parenchymal cells amplify IP-10 induction in primary human hepatocytes[145].

Next to IP-10, also the chemokine IL-8 was secreted (Fig. 4-10). To investigate if monocytes in fact secrete these chemokines, intracellular staining for IL-8 was performed after co-culture with HCV SGR cells (IL-8 was chosen as functioning antibodies for intracellular staining are available). Surprisingly, IL-8 was not secreted by monocytes but by HCV SGR cells (Fig. 4-13). Although previous reports have shown that viral proteins can induce IL-8 secretion in infected cells[146–148], IL-8 was not secreted by HCV SGR cells per se. Thus, PBMC activation seems to induce signaling in HCV SGR cells which leads to the secretion of IL-8. Thereby, IL-8 as a chemokine attracts further immune cells to the infected cell[149].

Despite no secretion of IL-8 by monocytes, intracellular IL-8 staining by chance revealed that monocytes take up material from HCV SGR cells (Fig. 4-13), finally indicating that monocytes indeed are involved in the anti-viral response.

5.3.2 Uptake of HCV SGR cells by monocytes

In co-cultures of CFSE-stained HCV SGR cells with PBMCs it was observed that nearly all monocytes became CFSE positive (Fig. 4-14). ImageStream analysis revealed that monocytes phagocytose small vesicles originating from HCV SGR cells (Fig. 4-15). These vesicles suggested that HCV SGR cells are driven into apoptosis by activated PBMCs. In fact, LDH release assays showed that HCV SGR cells are killed by PBMCs (Fig. 4-25 A). Moreover, caspase inhibition experiments (Fig. 4-26) and cleaved PARP assays (Fig. 4-25 B) demonstrated that PBMCs trigger apoptosis in HCV SGR cells. Thus, monocytes take up apoptotic vesicles from HCV SGR cells, which again raised the question if monocytes play an active role in the anti-viral response or if they only passively take up apoptotic vesicles. To answer this, purified monocytes were co-cultured with CFSE-stained HCV SGR cells. Also in this setup monocytes were taking up HCV SGR cells, although compared to monocytes within PBMCs to a lower extent (Fig. 4-19). However, time course experiments revealed that monocyte uptake occurs before interferons were secreted (Fig. 4-23). In addition, depletion of monocytes from PBMCs reduced killing activity of the remaining immune cells (Fig. 4-27 B). Taken together these results indicated that monocytes actively contribute to the response against HCV. Hence, this study confirms findings of an active role of monocytes, in which monocytes were shown to respond by inflammasome activation and IL-18 secretion in response to HCV infected cells[82].

5.4 Interaction of innate immune cells in the response against HCV

Previous results showed that pDCs, NK cells and monocytes are activated in co-culture with HCV SGR cells. Purified pDCs and pDCs within PBMCs secreted similar amounts of IFNα (Fig. 4-1), arguing for a direct recognition of HCV SGR cells by pDCs independent of other innate immune cells (see 5.1). Still, pDC activation alone was not sufficient to induce killing of HCV SGR cells (Fig. 4-27 A). Besides, purified NK cells and monocytes had impaired functions compared to their phenotype within PBMCs.

5.4.1 NK cell activation depends on monocytes and pDCs

Purified NK cells did not secrete IFNγ (Fig. 4-11 C) and failed to kill HCV SGR cells (Fig. 4-27 A). IFNγ production by NK cells was shown to be dependent on monocytes (Fig. 4-

24). As discussed above in detail (see. 5.2) this was in line with another report[82]). A further study suggested that pDCs are also important for IFNγ secretion by NK cells[102] (see 5.2). Moreover, NK cells were found to express the activation markers CD69 and CD25 and the apoptosis inducing ligand TRAIL on the surface in co-culture of HCV SGR cells with PBMCs (Fig. 4-28). Subsequently, it was shown that TRAIL induced apoptosis in HCV SGR cells, as blocking TRAIL by a specific antibody significantly reduced killing and uptake of HCV SGR cells (Fig. 4-29). Earlier reports have shown that expression of TRAIL by NK cells in the context of HCV infection depends on IFNα[103,104,150]. Hence, IFNα secretion by pDCs in this co-culture system is driving TRAIL expression on NK cells and thereby apoptosis of HCV SGR cells.

5.4.2 Monocyte activation is enhanced by pDCs and NK cells

Purified monocytes were impaired in uptake of HCV SGR cells compared to monocytes within PBMCs (Fig. 4-19), but still showed a significant response, which confirmed other studies that showed a direct recognition by monocytes of HCV infected cells[82]. Interestingly, monocyte uptake was not reduced when NK cells or pDCs were depleted from PBMC, but dropped significantly when both, NK cells and pDCs were depleted (Fig. 4-21). This indicated that pDCs and NK cells have redundant roles. Also addition of IFNα or IFNγ boosted the uptake of HCV SGR cells by purified monocytes (Fig. 4-22 A, B). Yet pre-incubation of monocytes or HCV SGR cells alone with interferon was not sufficient to boost the uptake, both cells had to be exposed to interferon during the co-culture (Fig. 4-22 C-F). This suggested that interferons act on monocytes as well as on HCV SGR cells. Nevertheless, this indicated that monocyte action is enhanced by pDCs and NK cells. In addition, an activation of monocytes by accessory cells is supported by the observation that monocytes within PBMCs showed strong up-regulation of activation markers CD80 and CD86 (Fig. 4-18), while purified monocytes displayed only slight up-regulation (Fig. 4-20).

A similar effect of pDCs and NK cells on monocytes has not been observed before in an HCV context. In a study dealing with pneumonia caused by influenza virus it was shown that type I interferons cause up-regulation of TRAIL on alveolar macrophages[151]. However, TRAIL was not detected on monocytes in this study, neither in the co-culture of HCV SGR cells with complete PBMCs nor with purified monocytes supplemented with IFNα (data not

shown). Also blocking TNF had no effect on the uptake by purified monocytes (data not shown). Another study in a murine model demonstrated that alveolar macrophages secrete type I interferons in response to the respiratory syncytial virus[152]. This triggered chemokine secretion by monocytes which in turn recruited inflammatory monocytes to the lungs. These inflammatory monocytes were then able to control viral infection. But similar to this study, the mechanism underlying elimination of infected cells could not be explained. Still, the data in this work provide evidence that pDCs and NK cells act on monocytes, which again highlights the importance of the interplay between innate immune cells.

5.4.3 Combined activation of innate immune cells leads to killing of HCV SGR cells

Complete PBMCs were able to kill HCV SGR cells (Fig. 4-25). However, purified pDCs, NK cells or monocytes were not sufficient to induce killing (Fig. 4-27 A). Nevertheless, each of these subsets of immune cells contributes to killing, as depletion of each of them resulted in lower killing efficiency of HCV SGR cells (Fig. 4-27 B). Again, this confirms the necessity for interactions of innate immune cells to initiate an immune response which is leading to killing and clearance of HCV SGR cells. Mechanistically, TRAIL expression on NK cells was found to induce apoptosis in HCV SGR cells (Fig. 4-28, Fig. 4-29). As discussed before, IFNγ secretion by NK cells depends on monocytes (Fig. 4-24) and TRAIL expression on IFNα[103,104]. The interactions between innate immune cells and how this leads to killing and clearance of HCV SGR cells are summarized in Fig. 5-1.

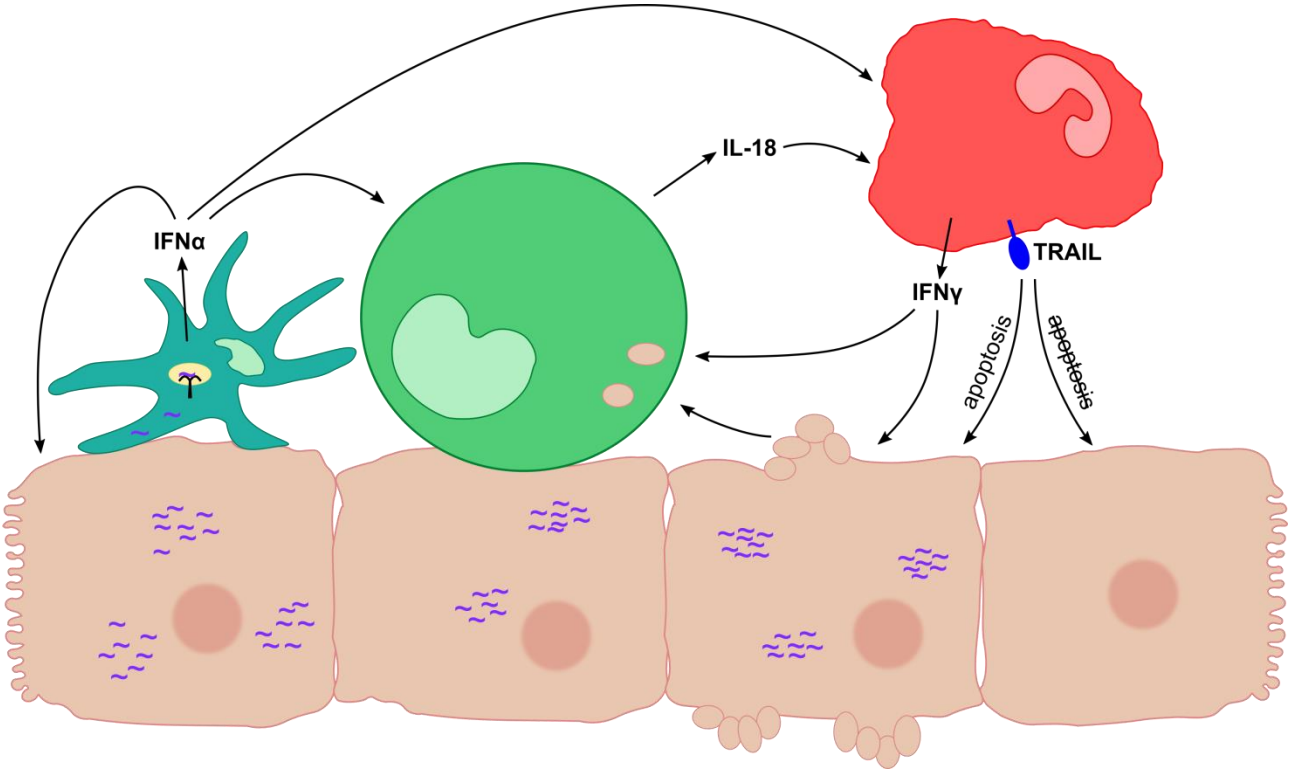

Fig. 5-1 Interaction of innate immune cells in response to HCV SGR cells
Schematic overview of the interactions of innate immune cells leading to anti-viral responses. pDC is depicted in cyan, monocyte in green, NK cell in red, viral RNA in purple and TLR7 inside the pDC in black. pDCs are activated by viral RNA in cell-cell contact dependent manner and secrete IFNα in response. IFNα inhibits viral replication and activates monocytes and NK cells, which leads to TRAIL expression on NK cells. Monocytes are also activated by HCV SGR cells. By secretion of IL-18 monocytes trigger IFNγ production in NK cells. IFNγ in turn also inhibits viral replication. TRAIL on NK cells induces apoptosis in HCV SGR cells, but not in uninfected cells. Moreover, IFNα from pDCs and IFNγ from NK cells act on monocytes and enhance a yet unknown mechanism by which monocytes kill HCV SGR cells. Finally, apoptotic bodies from HCV SGR cells are cleared by monocytes.

However, other mechanisms of killing are exerted by PBMCs within the co-culture of HCV SGR cells with PBMCs. This is supported by the results of NK depletion experiments. Upon NK depletion neither killing nor uptake of HCV SGR cells was completely lost (Fig. 4-27 B, Fig. 4-21). Moreover, purified monocytes in co-culture with HCV SGR cells in presence of interferons exerted some killing activity and the uptake was boosted (Fig. 4-22). Thus, activated monocytes are able to kill HCV SGR cells, since IFNα alone did not induce killing of HCV SGR cells (no kill by purified pDCs despite IFNα secretion, Fig. 4-27 A, Fig. 4-1 C). Although intensively investigated, the mechanism behind killing by monocytes could not be figured out. Monocytes are known to engage several mechanisms to kill target cells. For example, expression of Granzyme B[153], TNFα[154] or TRAIL[155,156] can lead to killing of target cells. Granzyme B expression was analyzed by qPCR in the co-

culture of purified monocytes and HCV SGR cells, but no up-regulation was detected (data not shown). Besides, neither TNF nor TRAIL blocking inhibited uptake by purified monocytes in presence of IFNα (data not shown). Thus, it remains unclear how monocytes kill HCV SGR cells. In other studies with HCV infected cells killing by monocytes thus far has not been described. As discussed before (see 5.4.2), studies with influenza virus have shown that TRAIL expression on monocytes in response to IFNα can kill infected cells[151], but TRAIL had no effect in this study (see above). Moreover, Goritzka et al. observed killing of respiratory syncytial virus infected cells by inflammatory monocytes in the murine system[152], but also struggled to identify the underlying mechanism. Nevertheless, it was proven that also the mechanism that enables monocytes to take up HCV SGR cells relies on interaction with other immune cells (Fig. 4-21, Fig. 4-22).

5.5 Specific killing of HCV infected cells

5.5.1 TRAIL in HCV infection

Similar to the observation in this study that TRAIL expressed on NK cells can induce apoptosis of HCV infected cells, in vitro studies with recombinant TRAIL showed that HCV infected cells can be killed by TRAIL[157,158]. However, it was also described that TRAIL unspecifically targets liver cells from HCV patients due to up-regulation of the pro apoptotic TRAIL receptors DR4 and DR5[159]. Conflicting with this was another study in which no up-regulation of DR4 and DR5 was found on hepatocytes in vivo during chronic hepatitis and with a study where no unspecific killing by TRAIL was induced in vivo[160,161]. The different results regarding DR4 and DR5 expression in patient samples might be explained by varying conditions in patients. For example, the infection status (acute or chronic) could have an influence on expression of TRAIL receptors. In addition, the immune response by each patient could inhibit or trigger expression of DR4 and DR5 via cytokine induced signaling. Also studies working with hepatoma cell lines replicating HCV had differential results regarding up-regulation of DR4 and DR5[157,158,162]. As cells lines are derived from a specific clone even the same cell lines often show different phenotypes, which of course is a major drawback of cell lines. On HCV SGR cells and Huh-7 control cells used in this study no differential expression in pro-apoptotic TRAIL receptors DR4 and DR5 and in the anti-apoptotic DcR2 receptor was detected (Fig. 4-30). Also flow cytometry measurements revealed no differences (data not shown).

5.5.2 HCV SGR cells are sensitive towards TRAIL

Since expression of TRAIL receptors between HCV SGR cells and Huh-7 cell was comparable, it was questionable if HCV activated PBMCs also target uninfected control cells. As discussed before, unspecific killing of uninfected cells *in vivo* would lead to harmful liver damage. To address this question, triple co-cultures of Huh-7, Huh-7 Con1 and PBMCs were set up. Strikingly, PBMCs did only kill HCV SGR cells, but not Huh-7 cells (Fig. 4-32, Fig. 4-33). Thus, TRAIL mediated killing in this experimental setup is specific for infected cells. However, this raised the question why killing is specific, as TRAIL receptors are not differentially expressed. It was found that recombinant TRAIL induced apoptosis only in HCV SGR cells (Fig. 4-36 A, C, D). This effect was specific for TRAIL, as TNF had no significant effect (Fig. 4-36 B).

In a former study it was shown, that sensitization towards TRAIL is a result of mitochondrial damage in HCV infected cells[158]. The authors showed that mitochondrial damage is caused by non-structural proteins of HCV and that caspase-9 signaling occurs. Capase-9 is activated by mitochondrial damage and mediates the intrinsic apoptosis pathway[163]. It was shown that an external apoptotic signal is not sufficient to drive apoptosis in hepatocytes, but that a second internal apoptotic signal like mitochondrial damage is required[164]. Thus, together with the extrinsic induction of apoptosis via TRAIL, the infected cell undergoes apoptosis. Thereby, it can be explained why in this study in the model of acute infection only HCV SGR cells are targeted. Nevertheless, in a chronic HCV infection *in vivo* it is conceivable that also uninfected cells become sensitive to external apoptosis induction due to the constant exposition to various cytokines, which could result in unspecific liver damage. Regarding the mitochondrial damage in HCV infected cells observed by Lan et al. [158], it is tempting to speculate that mitochondrial damage arises from MAVS cleavage. MAVS is a mitochondrial membrane protein involved in RLR signaling[165] and can be cleaved by the viral NS3/4A protease[48]. This hypothesis could be tested by analyzing if cells expressing a cleavage-resistant MAVS mutant are also sensitive to TRAIL induced apoptosis.

In addition to specific killing due to TRAIL sensitization, it is speculated that specificity for infected cells might also arise from direct interactions of innate immune cells. Potentially, NK cells might only be activated at sites where pDCs and monocytes are activated by direct contact with HCV infected cells. Thus, the interaction of innate immune cells might increase specificity by coupling recognition of infected cells and effector mechanisms. This

hypothesis to a certain degree is supported by the observation that NK cells displayed stronger adherence to HCV SGR cells compared to Huh-7 control cells (Fig. 4-34). This speculated process might function similar to the complement system, the humoral part of the innate immune system (reviewed in Merle et al.[166]). In brief, pathogens are marked by the complement system by various mechanisms, which in turn leads to specific targeting of the marked pathogens e.g. by opsonization. In the context of this study, pDCs and monocytes sense HCV infected cells by direct contact and respond by cytokine secretion. In addition, it was observed that immune cells drive chemokine secretion by HCV SGR cells (Fig. 4-13). This high local concentration of cytokines might act as a marker for an ongoing infection and could lead to site-specific activation of NK cells. Thereby, NK cells could specifically target infected cells. It is currently investigated if this hypothesized interaction between innate immune cells takes place in the liver of HCV infected patients. Therefore, monocytes and NK cells are stained in liver slides and analyzed for co-localization.

5.5.3 PBMCs are specifically activated by HCV, but not by DV and HAV SGR cells

Next to specificity in killing HCV SGR cells without targeting uninfected cells, activation of PBMCs was specific for HCV. DV and HAV SGR cells were not taken up by monocytes (Fig. 4-17), suggesting that PBMCs are not activated in this setting. Indeed, both types of SGR cells did not induce IFNα secretion and supernatants of the co-culture with PBMCs did not inhibit viral replication in the highly sensitive luciferase assay (data not shown). It was shown that IFNα secretion correlated with the amount of HCV RNA secreted from different HCV SGR cells (Fig. 4-3). DV and HAV SGR cells secreted lower amounts of viral RNA compared to HCV (data not shown). Hence, PBMCs are not activated by SGR cells replicating DV or HAV RNA. In addition, due to GU-rich sequences HCV RNA can be recognized by TLR7 and TLR8[120,128], which could also explain why innate immune cells react to HCV, but not to DV or HAV SGR cells. Furthermore, it has been shown that immature viral particles of DV stimulated pDCs[167]. In another study it was demonstrated that pDCs sense enveloped virions of HAV[168]. As SGR cells lack structural proteins no particles can be produced, which might explain the lack in PBMC activation with DV and HAV SGR cells. Interestingly, both studies observed that pDC activation required direct contact with infected cells, which as discussed before is in line with findings for HCV.

To test if DV and HAV RNA activate PBMCs, RNA from these viruses could be transfected into PBMCs. If indeed a sequence specific recognition leads to a response against HCV RNA, DV and HAV RNA should not activate PBMCs. However, it has been shown that modifications of RNAs can also inhibit stimulation of immune cells[169]. Thus, transfection experiments of viral RNA should be performed with in vitro transcripts lacking modifications and with purified viral RNA from host cells that carry different modifications. If also DV and HAV RNA would stimulate immune cells, this would indicate that indeed the reduced secretion of viral RNA compared to HCV accounts for the lack of PBMC stimulation.

Moreover, it could be analyzed if DV or HAV SGR cells are killed by PBMCs when recombinant IFNα and/or IFNγ is added to the co-culture. Alternatively, triple co-cultures of PBMCs with HCV and DV or HAV SGR cells could be set up. That would give insight if DV and HAV SGR cells are killed by activated PBMCs and if DV and HAV similar to HCV sensitize host cells for TRAIL mediated apoptosis.

5.6 Innate immunity and the outcome of HCV infection

With the experimental setup in this study, the co-culture of HCV SGR cells with PBMCs from healthy donors, an acute infection is mimicked. Indeed, the results clearly show that innate immune cells elicit an anti-viral response that leads to killing and clearance of infected cells. Hence, it is remarkable why 70-80% of HCV infections lead to chronic infection while only 20-30% spontaneously clear the virus[170]. Other reports provided evidence that the adaptive immune response and cytotoxic T cells in particular determine the outcome of HCV infection (reviewed in[171]). Due the short time frame of the experiments performed in this study adaptive immunity had no influence. Nevertheless, it might be speculated that the interaction and mutual activation of innate immune cells might also influence the onset and magnitude of the adaptive immune response.

The results of this study demonstrated that the innate immune response rapidly leads to apoptosis and clearance of infected cells. Via uptake of apoptotic bodies of HCV infected cells, antigen presenting cells might be able to display HCV specific antigens to cells of the adaptive immune system. Moreover, activation of antigen presenting cells in the context of the anti-viral response might also lead to up-regulation of co-stimulatory molecules, which

are essential in activating T cells. Yet, an efficient innate immune response relies on interaction of different immune cells and it is questionable if these interactions *in vivo* occur as fast as in this *in vitro* model. Thus, it could be speculated that a race between the immune response and the establishment of infection takes place in which HCV inhibits several immune responses. In case of a fast response innate immune cells could rapidly activate adaptive immune cells that finally clear the virus, before HCV exerts its immune-modulatory functions. However, it might also be speculated that a strong innate immune response could limit adaptive immunity. By killing and clearance of HCV infected cells, the number of infected cells is kept at a low percentage, which in turn could help the virus to establish a chronic infection below the radar of adaptive immunity.

5.7 Conclusion & Outlook

In summary, in this work it is shown how interaction of different innate immune cells in response to HCV infected cells shapes the immune response (summarized in Fig 5-1). While pDCs and monocytes are directly activated by recognition of viral RNA, the interaction facilitates additional mutual activation of innate immune cells. Consequently, an efficient immune response is exerted that ultimately leads to killing of HCV infected cells by TRAIL expression on NK cells and other thus far unidentified mechanisms. In addition, it was demonstrated that innate immune cells activated by HCV in this acute infection model do not target uninfected cells. Nevertheless, some important aspects were not addressed in this study or could not be answered yet. Thus, additional experiments could be performed to further understand the innate immune response against HCV.

Whether similar interactions between innate immune cells also occur in the infected liver is subject of current analysis. Co-localization of NK cells and monocytes is investigated in liver tissue slides from HCV patients. However, a direct co-localization is not necessarily essential, as the results of this and other studies indicated that mutual activation also relies on cytokine secretion.

An additional interesting topic that arose from the results of this study is to investigate the mechanism underlying transfer of viral RNA from infected cells into pDCs. It was demonstrated that pDC activation was dependent on direct cell-cell contact with HCV SGR cells (Fig. 4-8). As discussed before (see 5.1.2) it is speculated that a synapse between

infected cells and pDCs is formed that allows transfer of HCV RNA into pDCs. To experimentally test this assumption, microscopy studies could be performed to analyze if such a synapse is formed and to identify molecular players involved in this contact by fluorescently tagged proteins or antibodies (which however could also disturb potential interactions). It would also be interesting to analyze if pDCs display stronger binding to HCV SGR cells compared to Huh-7 control cells (for example by atomic force microscopy). Although no major differences were found in the surface proteome of these cells (Fig. 4-35), it is still possible that pDCs somehow recognize infected cells from the outside. If binding to HCV SGR cells and control cells would be of similar strength, it would indicate that infected cells are not recognized from the outside.

Next to further analysis regarding pDCs, it remains unclear how purified monocytes in co-culture with HCV SGR cells in presence of IFNα or IFNγ mediate killing of infected cells (Fig. 4-22). Thus far, a role of Granzyme B, TRAIL and TNFα was excluded. Another option could be the analysis of the gene expression in purified monocytes upon stimulation with interferons by microarray. Thereby, potential new candidates might be found that could explain how monocytes kill HCV SGR cells. An effect of these candidates then could be analyzed by knockdown or blocking experiments.

At last, it should be experimentally investigated if the interaction of innate immune cells is beneficial for the activation of adaptive immune cells. This could be analyzed by setting up the co-culture of HCV SGR cells and PBMCs. Subsequently, different populations (monocytes, pDCs and classical DCs) could be purified. Purified cells could then be co-cultured with T cells to analyze if T cells become activated by HCV specific antigens presented by innate immune cells (that were in contact with HCV SGR cells). Alternatively, after co-culture with HCV SGR cells purified innate immune cells could also be spiked with tetanus antigen (which most people are vaccinated against). Then it could be analyzed if T cells show a stronger response in co-culture with innate immune cells that were previously co-cultured with HCV SGR cells compared to Huh-7 control cells. That at least would indicate that exposure to HCV SGR cells increases the co-stimulatory potential of innate immune cells. If these experiments are feasible, the initial co-culture with HCV SGR cells could be performed with purified innate immune cells or with PBMCs depleted from specific subsets. If T cell activation in that case would be impaired it would indicate that the interaction of innate immune cells is also required for an efficient activation of adaptive immunity.

The activation of adaptive immunity by innate immune cells could also be analyzed in a murine model. In this work it was shown that murine BMDM respond to HCV SGR cells (Fig. 4-5). In addition, T cells from OT-I mice that express a T cell receptor that recognizes ovalbumin presented by MCH class I could be used[172]. Then it could be tested if BMDM activated by HCV SGR cells and pulsed with ovalbumin induce a stronger T cell response compared to BMDM that were cultured with hepatoma cells without HCV replication. However, a drawback of the murine model would be the missing interactions between innate immune cells. It is not known if other cell types in the murine system respond to HCV SGR cells and it would require additional work to investigate this.

To conclude, this work demonstrates that interacting innate immune cells respond to HCV and provide a rapid and efficient response, but further details of the innate immune response need to be investigated. And finally it needs to be analyzed how the innate immune response influences adaptive immunity to better understand why some patients clear the virus and the majority develops a chronic infection.

6 Bibliography

1. Medzhitov R, Janeway CA. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997; 91:295–8.

2. Medzhitov R, Janeway CA. Innate immune recognition and control of adaptive immune responses. Semin Immunol 1998; 10:351–3.

3. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB. Phylogeny of the genus Flavivirus. J Virol 1998; 72:73–83.

4. Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 2000; 20:1–16.

5. Lavanchy D. Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 2011; 17:107–15.

6. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich H-G, Mizokami M, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005; 11:791–6.

7. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nat Rev Microbiol 2007; 5:453–63.

8. Bartenschlager R, Frese M, Pietschmann T. Novel insights into hepatitis C virus replication and persistence. Adv Virus Res 2004; 63:71–180.

9. Bartenschlager R, Penin F, Lohmann V, André P. Assembly of infectious hepatitis C virus particles. Trends Microbiol 2011; 19:95–103.

10. Moradpour D, Penin F. Hepatitis C virus proteins: from structure to function. Curr Top Microbiol Immunol 2013; 369:113–42.

11. Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatol Baltim Md 2005; 42:962–73.

12. Poynard T, Yuen M-F, Ratziu V, Lai CL. Viral hepatitis C. Lancet Lond Engl 2003; 362:2095–100.

13. Ghany MG, Strader DB, Thomas DL, Seeff LB, American Association for the Study of Liver Diseases. Diagnosis, management, and treatment of hepatitis C: an update. Hepatol Baltim Md 2009; 49:1335–74.

14. Ferenci P, Reddy KR. Impact of HCV protease-inhibitor-based triple therapy for chronic HCV genotype 1 infection. Antivir Ther 2011; 16:1187–201.

15. Ghany MG, Liang TJ. Current and future therapies for hepatitis C virus infection. N Engl J Med 2013; 369:679–80.

16. Childs-Kean LM, Hand EO. Simeprevir and Sofosbuvir for Treatment of Chronic Hepatitis C Infection. Clin Ther 2015; 37:243–67.

17. Lavillette D, Tarr AW, Voisset C, Donot P, Bartosch B, Bain C, Patel AH, Dubuisson J, Ball JK, Cosset F-L. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. Hepatol Baltim Md 2005; 41:265–74.

18. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, et al. Binding of hepatitis C virus to CD81. Science 1998; 282:938–41.

19. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wölk B, Hatziioannou T, McKeating JA, Bieniasz PD, Rice CM. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 2007; 446:801–5.

20. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 2009; 457:882–6.

21. Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C, Rouillé Y. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J Virol 2006; 80:6964–72.

22. Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A 2003; 100:7271–6.

23. Romero-Brey I, Merz A, Chiramel A, Lee J-Y, Chlanda P, Haselman U, Santarella-Mellwig R, Habermann A, Hoppe S, Kallis S, et al. Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication. PLoS Pathog 2012; 8:e1003056.

24. Targett-Adams P, Boulant S, McLauchlan J. Visualization of double-stranded RNA in cells supporting hepatitis C virus RNA replication. J Virol 2008; 82:2182–95.

25. Binder M, Kochs G, Bartenschlager R, Lohmann V. Hepatitis C virus escape from the interferon regulatory factor 3 pathway by a passive and active evasion strategy. Hepatol Baltim Md 2007; 46:1365–74.

26. Pause A, Kukolj G, Bailey M, Brault M, Dô F, Halmos T, Lagacé L, Maurice R, Marquis M, McKercher G, et al. An NS3 Serine Protease Inhibitor Abrogates Replication of Subgenomic Hepatitis C Virus RNA. J Biol Chem 2003; 278:20374–80.

27. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, Strand D, Bartenschlager R. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002; 76:4008–21.

28. Lohmann V, Körner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999; 285:110–3.

29. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 1982; 42:3858–63.

30. Cheng G, Zhong J, Chisari FV. Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. Proc Natl Acad Sci U S A 2006; 103:8499–504.

31. Lohmann V, Körner F, Dobierzewska A, Bartenschlager R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J Virol 2001; 75:1437–49.

32. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, Wakita T. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 2003; 125:1808–17.

33. Friebe P, Boudet J, Simorre J-P, Bartenschlager R. Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. J Virol 2005; 79:380–92.

34. Bartenschlager R. Hepatitis C virus replicons: potential role for drug development. Nat Rev Drug Discov 2002; 1:911–6.

35. Matzinger P. The danger model: a renewed sense of self. Science 2002; 296:301–5.

36. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 2006; 440:228–32.

37. Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, Mudd S, Mann N, Sovath S, Goode J, et al. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol 2006; 7:156–64.

38. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001; 413:732–8.

39. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004; 303:1529–31.

40. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004; 303:1526–9.

41. Pichlmair A, Schulz O, Tan CP, Näslund TI, Liljeström P, Weber F, Reis e Sousa C. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science 2006; 314:997–1001.

42. Binder M, Eberle F, Seitz S, Mücke N, Hüber CM, Kiani N, Kaderali L, Lohmann V, Dalpke A, Bartenschlager R. Molecular mechanism of signal perception and integration by the innate immune sensor retinoic acid-inducible gene-I (RIG-I). J Biol Chem 2011; 286:27278–87.

43. Yue M, Gao C, Wang J, Wang C, Feng L, Wang J, Yu R, Peng Z, Xue X, Cai L, et al. Toll-like receptor 7 variations are associated with the susceptibility to HCV infection among Chinese females. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis 2014; 27:264–70.

44. Fernández-Rodríguez A, Berenguer J, Jiménez-Sousa MA, García–Álvarez M, Aldámiz-Echevarría T, Pineda-Tenor D, Diez C, de la Barrera J, Bellon JM, Briz V, et al. Toll-like receptor 8 (TLR8) polymorphisms are associated with non-progression of chronic hepatitis C in HIV/HCV coinfected patients. Infect Genet Evol 2015; 36:339–44.

45. Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature 2008; 454:523–7.

46. Yoneyama M, Fujita T. Recognition of viral nucleic acids in innate immunity. Rev Med Virol 2010; 20:4–22.

47. Sadler AJ, Williams BRG. Interferon-inducible antiviral effectors. Nat Rev Immunol 2008; 8:559–68.

48. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 2005; 437:1167–72.

49. Li K, Foy E, Ferreon JC, Nakamura M, Ferreon ACM, Ikeda M, Ray SC, Gale M, Lemon SM. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci U S A 2005; 102:2992–7.

50. Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR, Luxon BA, Lemon SM, Lanford RE. Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. J Virol 2004; 78:13779–92.

51. Alter HJ, Seeff LB. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin Liver Dis 2000; 20:17–35.

52. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, Liu YJ. The nature of the principal type 1 interferon-producing cells in human blood. Science 1999; 284:1835–7.

53. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 2001; 194:863–9.

54. Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol 2001; 31:3388–93.

55. Lau DT-Y, Fish PM, Sinha M, Owen DM, Lemon SM, Gale M. Interferon regulatory factor-3 activation, hepatic interferon-stimulated gene expression, and immune cell infiltration in hepatitis C virus patients. Hepatology 2008; 47:799–809.

56. Kanto T, Inoue M, Miyatake H, Sato A, Sakakibara M, Yakushijin T, Oki C, Itose I, Hiramatsu N, Takehara T, et al. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. J Infect Dis 2004; 190:1919–26.

57. Shiina M, Rehermann B. Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function. Hepatol Baltim Md 2008; 47:385–95.

58. Gondois-Rey F, Dental C, Halfon P, Baumert TF, Olive D, Hirsch I. Hepatitis C Virus Is a Weak Inducer of Interferon Alpha in Plasmacytoid Dendritic Cells in Comparison with Influenza and Human Herpesvirus Type-1. PLoS ONE 2009; 4:e4319.

59. Takahashi K, Asabe S, Wieland S, Garaigorta U, Gastaminza P, Isogawa M, Chisari FV. Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. Proc Natl Acad Sci 2010; 107:7431–6.

60. Masciopinto F, Giovani C, Campagnoli S, Galli-Stampino L, Colombatto P, Brunetto M, Yen TSB, Houghton M, Pileri P, Abrignani S. Association of hepatitis C virus envelope proteins with exosomes. Eur J Immunol 2004; 34:2834–42.

61. Ludwig A-K, Giebel B. Exosomes: small vesicles participating in intercellular communication. Int J Biochem Cell Biol 2012; 44:11–5.

62. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007; 9:654–9.

63. Dreux M, Garaigorta U, Boyd B, Décembre E, Chung J, Whitten-Bauer C, Wieland S, Chisari FV. Short-Range Exosomal Transfer of Viral RNA from Infected Cells to Plasmacytoid Dendritic Cells Triggers Innate Immunity. Cell Host Microbe 2012; 12:558–70.

64. Février B, Raposo G. Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr Opin Cell Biol 2004; 16:415–21.

65. Tamai K, Tanaka N, Nakano T, Kakazu E, Kondo Y, Inoue J, Shiina M, Fukushima K, Hoshino T, Sano K, et al. Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein. Biochem Biophys Res Commun 2010; 399:384–90.

66. Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, Ivarsson Y, Depoortere F, Coomans C, Vermeiren E, et al. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol 2012; 14:677–85.

67. Filipenko NR, MacLeod TJ, Yoon C-S, Waisman DM. Annexin A2 is a novel RNA-binding protein. J Biol Chem 2004; 279:8723–31.

68. Aukrust I, Hollås H, Strand E, Evensen L, Travé G, Flatmark T, Vedeler A. The mRNA-binding site of annexin A2 resides in helices C-D of its domain IV. J Mol Biol 2007; 368:1367–78.

69. Mayran N, Parton RG, Gruenberg J. Annexin II regulates multivesicular endosome biogenesis in the degradation pathway of animal cells. EMBO J 2003; 22:3242–53.

70. Gerke V, Creutz CE, Moss SE. Annexins: linking Ca2+ signalling to membrane dynamics. Nat Rev Mol Cell Biol 2005; 6:449–61.

71. Morel E, Parton RG, Gruenberg J. Annexin A2-dependent polymerization of actin mediates endosome biogenesis. Dev Cell 2009; 16:445–57.

72. Backes P, Quinkert D, Reiss S, Binder M, Zayas M, Rescher U, Gerke V, Bartenschlager R, Lohmann V. Role of annexin A2 in the production of infectious hepatitis C virus particles. J Virol 2010; 84:5775–89.

73. Saxena V, Lai C-K, Chao T-C, Jeng K-S, Lai MMC. Annexin A2 is involved in the formation of hepatitis C virus replication complex on the lipid raft. J Virol 2012; 86:4139–50.

74. Ziegler-Heitbrock HW, Ulevitch RJ. CD14: cell surface receptor and differentiation marker. Immunol Today 1993; 14:121–5.

75. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJM, Liu Y-J, MacPherson G, Randolph GJ, et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010; 116:e74-80.

76. Wong KL, Tai JJ-Y, Wong W-C, Han H, Sem X, Yeap W-H, Kourilsky P, Wong S-C. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood 2011; 118:e16-31.

77. Yasaka T, Mantich NM, Boxer LA, Baehner RL. Functions of human monocyte and lymphocyte subsets obtained by countercurrent centrifugal elutriation: differing functional capacities of human monocyte subsets. J Immunol Baltim Md 1950 1981; 127:1515–8.

78. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G, Geissmann F. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 2007; 317:666–70.

79. Epelman S, Lavine KJ, Randolph GJ. Origin and Functions of Tissue Macrophages. Immunity 2014; 41:21–35.

80. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, Endres S, Hartmann G. Quantitative Expression of Toll-Like Receptor 1–10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides. J Immunol 2002; 168:4531–7.

81. Revie D. Role of macrophages and monocytes in hepatitis C virus infections. World J Gastroenterol 2014; 20:2777.

82. Serti E, Werner JM, Chattergoon M, Cox AL, Lohmann V, Rehermann B. Monocytes Activate Natural Killer Cells via Inflammasome-Induced Interleukin 18 in Response to Hepatitis C Virus Replication. Gastroenterology 2014; 147:209–220.e3.

83. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med 1989; 320:1731–5.

84. Orange JS. Human natural killer cell deficiencies and susceptibility to infection. Microbes Infect 2002; 4:1545–58.

85. Kiessling R, Klein E, Wigzell H. “Natural” killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol 1975; 5:112–7.

86. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. Int J Cancer 1975; 16:216–29.

87. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 1986; 319:675–8.

88. Brandstadter JD, Yang Y. Natural Killer Cell Responses to Viral Infection. J Innate Immun 2011; 3:274–9.

89. Orange S. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. J Exp Med 1995; 182:1045–56.

90. Natuk RJ, Welsh RM. Accumulation and chemotaxis of natural killer/large granular lymphocytes at sites of virus replication. J Immunol Baltim Md 1950 1987; 138:877–83.

91. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 2003; 3:361–70.

92. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 1995; 3:673–82.

93. Pan G, O’Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM. The receptor for the cytotoxic ligand TRAIL. Science 1997; 276:111–3.

94. Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, Timour MS, Gerhart MJ, Schooley KA, Smith CA, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 1997; 16:5386–97.

95. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 1997; 7:813–20.

96. Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P, et al. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr Biol CB 1997; 7:1003–6.

97. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, von Goetz M, Yee SF, Totpal K, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med 2007; 13:1070–7.

98. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 2003; 22:8628–33.

99. Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 2010; 29:4752–65.

100. Oliver FJ, de la Rubia G, Rolli V, Ruiz-Ruiz MC, de Murcia G, Murcia JM. Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. J Biol Chem 1998; 273:33533–9.

101. Larkin J, Bost A, Glass JI, Tan S-L. Cytokine-activated natural killer cells exert direct killing of hepatoma cells harboring hepatitis C virus replicons. J Interferon Cytokine Res 2006; 26:854–865.

102. Zhang S, Saha B, Kodys K, Szabo G. IFN-γ production by human natural killer cells in response to HCV-infected hepatoma cells is dependent on accessory cells. J Hepatol 2013; 59:442–449.

103. Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, Ghany MG, Hoofnagle JH, Liang TJ, Heller T, et al. Natural Killer Cells Are Polarized Toward Cytotoxicity in Chronic Hepatitis C in an Interferon-Alfa–Dependent Manner. Gastroenterology 2010; 138:325–335.e2.

104. Stegmann KA, Björkström NK, Veber H, Ciesek S, Riese P, Wiegand J, Hadem J, Suneetha PV, Jaroszewicz J, Wang C, et al. Interferon-α–Induced TRAIL on Natural Killer Cells Is Associated With Control of Hepatitis C Virus Infection. Gastroenterology 2010; 138:1885–1897.e10.

105. Lohmann V, Hoffmann S, Herian U, Penin F, Bartenschlager R. Viral and Cellular Determinants of Hepatitis C Virus RNA Replication in Cell Culture. J Virol 2003; 77:3007–19.

106. Kumar A, Buhler S, Selisko B, Davidson A, Mulder K, Canard B, Miller S, Bartenschlager R. Nuclear Localization of Dengue Virus Nonstructural Protein 5 Does Not Strictly Correlate with Efficient Viral RNA Replication and Inhibition of Type I Interferon Signaling. J Virol 2013; 87:4545–57.

107. Esser-Nobis K, Harak C, Schult P, Kusov Y, Lohmann V. Novel perspectives for hepatitis A virus therapy revealed by comparative analysis of hepatitis C virus and hepatitis A virus RNA replication. Hepatology 2015; 62:397–408.

108. Tanaka M, Kawamura K, Fang M, Higashino K, Kishimoto S, Nakabayashi H, Sato J. Production of fibronectin by HUH6 C15 cell line established from a human hepatoblastoma. Biochem Biophys Res Commun 1983; 110:837–41.

109. Grünvogel O, Esser-Nobis K, Reustle A, Schult P, Müller B, Metz P, Trippler M, Windisch MP, Frese M, Binder M, et al. DDX60L Is an Interferon-Stimulated Gene Product Restricting Hepatitis C Virus Replication in Cell Culture. J Virol 2015; 89:10548–68.

110. Blight KJ, McKeating JA, Rice CM. Highly Permissive Cell Lines for Subgenomic and Genomic Hepatitis C Virus RNA Replication. J Virol 2002; 76:13001–14.

111. Kress S, König J, Schweizer J, Löhrke H, Bauer-Hofmann R, Schwarz M. p53 mutations are absent from carcinogen-induced mouse liver tumors but occur in cell lines established from these tumors. Mol Carcinog 1992; 6:148–58.

112. Boltz-Nitulescu G, Wiltschke C, Holzinger C, Fellinger A, Scheiner O, Gessl A, Förster O. Differentiation of rat bone marrow cells into macrophages under the influence of mouse L929 cell supernatant. J Leukoc Biol 1987; 41:83–91.

113. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset F-L, et al. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci 2006; 103:7408–7413.

114. Häcker H, Mischak H, Miethke T, Liptay S, Schmid R, Sparwasser T, Heeg K, Lipford GB, Wagner H. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. EMBO J 1998; 17:6230–40.

115. Sioud M. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. J Mol Biol 2005; 348:1079–90.

116. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brügger B, Simons M. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 2008; 319:1244–7.

117. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, Moita CF, Schauer K, Hume AN, Freitas RP, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol 2010; 12:19-30; sup pp 1-13.

118. Zhang S, Kodys K, Babcock GJ, Szabo G. CD81/CD9 tetraspanins aid plasmacytoid dendritic cells in recognition of hepatitis C virus–infected cells and induction of interferon-alpha. Hepatology 2013; 58:940–949.

119. Heinz LX, Baumann CL, Köberlin MS, Snijder B, Gawish R, Shui G, Sharif O, Aspalter IM, Müller AC, Kandasamy RK, et al. The Lipid-Modifying Enzyme SMPDL3B Negatively Regulates Innate Immunity. Cell Rep 2015; 11:1919–28.

120. Zhang Y-L, Guo Y-J, Bin Li, Sun S-H. Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7. J Hepatol 2009; 51:29–38.

121. Nishiya T, DeFranco AL. Ligand-regulated Chimeric Receptor Approach Reveals Distinctive Subcellular Localization and Signaling Properties of the Toll-like Receptors. J Biol Chem 2004; 279:19008–17.

122. Liu J, Xu C, Hsu L-C, Luo Y, Xiang R, Chuang T-H. A five-amino-acid motif in the undefined region of the TLR8 ectodomain is required for species-specific ligand recognition. Mol Immunol 2010; 47:1083–90.

123. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408:740–5.

124. Hidmark A, von Saint Paul A, Dalpke AH. Cutting edge: TLR13 is a receptor for bacterial RNA. J Immunol Baltim Md 1950 2012; 189:2717–21.

125. Li K, Li NL, Wei D, Pfeffer SR, Fan M, Pfeffer LM. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. Hepatol Baltim Md 2012; 55:666–75.

126. Wang Y, Li J, Wang X, Zhou Y, Zhang T, Ho W. HCV dsRNA-Activated Macrophages Inhibit HCV Replication in Hepatocytes. Hepat Mon 2015; 15:e29282.

127. Zhou Y, Wang X, Sun L, Zhou L, Ma T-C, Song L, Wu J-G, Li J-L, Ho W-Z. Toll-like receptor 3-activated macrophages confer anti-HCV activity to hepatocytes through exosomes. FASEB J Off Publ Fed Am Soc Exp Biol 2016;

128. Zhang Y, El-Far M, Dupuy FP, Abdel-Hakeem MS, He Z, Procopio FA, Shi Y, Haddad EK, Ancuta P, Sekaly R-P, et al. HCV RNA Activates APCs via TLR7/TLR8 While Virus Selectively Stimulates Macrophages Without Inducing Antiviral Responses. Sci Rep 2016; 6:29447.

129. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 2010; 285:17442–52.

130. Silvie O, Charrin S, Billard M, Franetich J-F, Clark KL, van Gemert G-J, Sauerwein RW, Dautry F, Boucheix C, Mazier D, et al. Cholesterol contributes to the organization of tetraspanin-enriched microdomains and to CD81-dependent infection by malaria sporozoites. J Cell Sci 2006; 119:1992–2002.

131. Marukian S, Jones CT, Andrus L, Evans MJ, Ritola KD, Charles ED, Rice CM, Dustin LB. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatol Baltim Md 2008; 48:1843–50.

132. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. The immunological synapse: a molecular machine controlling T cell activation. Science 1999; 285:221–7.

133. Webster B, Assil S, Dreux M. Cell-Cell Sensing of Viral Infection by Plasmacytoid Dendritic Cells. J Virol 2016; 90:10050–3.

134. Deaglio S, Morra M, Mallone R, Ausiello CM, Prager E, Garbarino G, Dianzani U, Stockinger H, Malavasi F. Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. J Immunol 1998; 160:395–402.

135. Kim S-J, Kim J-S, Papadopoulos J, Wook Kim S, Maya M, Zhang F, He J, Fan D, Langley R, Fidler IJ. Circulating Monocytes Expressing CD31. Am J Pathol 2009; 174:1972–80.

136. Tu Z, Bozorgzadeh A, Pierce RH, Kurtis J, Crispe IN, Orloff MS. TLR-dependent cross talk between human Kupffer cells and NK cells. J Exp Med 2008; 205:233–44.

137. Lau DT-Y, Negash A, Chen J, Crochet N, Sinha M, Zhang Y, Guedj J, Holder S, Saito T, Lemon SM, et al. Innate immune tolerance and the role of kupffer cells in differential responses to interferon therapy among patients with HCV genotype 1 infection. Gastroenterology 2013; 144:402–413.e12.

138. Jovinge S, Ares MP, Kallin B, Nilsson J. Human monocytes/macrophages release TNF-alpha in response to Ox-LDL. Arterioscler Thromb Vasc Biol 1996; 16:1573–9.

139. Lukacs NW, Strieter RM, Elner V, Evanoff HL, Burdick MD, Kunkel SL. Production of chemokines, interleukin-8 and monocyte chemoattractant protein-1, during monocyte: endothelial cell interactions. Blood 1995; 86:2767–73.

140. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. J Interferon Cytokine Res 2009; 29:313–26.

141. Standiford TJ, Kunkel SL, Liebler JM, Burdick MD, Gilbert AR, Strieter RM. Gene expression of macrophage inflammatory protein-1 alpha from human blood monocytes and alveolar macrophages is inhibited by interleukin-4. Am J Respir Cell Mol Biol 1993; 9:192–8.

142. Harvey CE, Post JJ, Palladinetti P, Freeman AJ, Ffrench RA, Kumar RK, Marinos G, Lloyd AR. Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. J Leukoc Biol 2003; 74:360–9.

143. Helbig KJ, Ruszkiewicz A, Lanford RE, Berzsenyi MD, Harley HA, McColl SR, Beard MR. Differential expression of the CXCR3 ligands in chronic hepatitis C virus (HCV) infection and their modulation by HCV in vitro. J Virol 2009; 83:836–46.

144. Zeremski M, Petrovic LM, Talal AH. The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection. J Viral Hepat 2007; 14:675–87.

145. Brownell J, Wagoner J, Lovelace ES, Thirstrup D, Mohar I, Smith W, Giugliano S, Li K, Crispe IN, Rosen HR, et al. Independent, parallel pathways to CXCL10 induction in HCV-infected hepatocytes. J Hepatol 2013; 59:701–8.

146. Chen W-C, Tseng C-K, Chen Y-H, Lin C-K, Hsu S-H, Wang S-N, Lee J-C. HCV NS5A Up-Regulates COX-2 Expression via IL-8-Mediated Activation of the ERK/JNK MAPK Pathway. PloS One 2015; 10:e0133264.

147. Polyak SJ, Khabar KS, Paschal DM, Ezelle HJ, Duverlie G, Barber GN, Levy DE, Mukaida N, Gretch DR. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. J Virol 2001; 75:6095–106.

148. Kadoya H, Nagano-Fujii M, Deng L, Nakazono N, Hotta H. Nonstructural proteins 4A and 4B of hepatitis C virus transactivate the interleukin 8 promoter. Microbiol Immunol 2005; 49:265–73.

149. Mukaida N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. Int J Hematol 2000; 72:391–8.

150. Varchetta S, Mele D, Mantovani S, Oliviero B, Cremonesi E, Ludovisi S, Michelone G, Alessiani M, Rosati R, Montorsi M, et al. Impaired intrahepatic natural killer cell cytotoxic function in chronic hepatitis C virus infection. Hepatol Baltim Md 2012; 56:841–9.

151. Högner K, Wolff T, Pleschka S, Plog S, Gruber AD, Kalinke U, Walmrath H-D, Bodner J, Gattenlöhner S, Lewe-Schlosser P, et al. Macrophage-expressed IFN-β Contributes to Apoptotic Alveolar Epithelial Cell Injury in Severe Influenza Virus Pneumonia. PLoS Pathog 2013; 9:e1003188.

152. Goritzka M, Makris S, Kausar F, Durant LR, Pereira C, Kumagai Y, Culley FJ, Mack M, Akira S, Johansson C. Alveolar macrophage-derived type I interferons orchestrate innate immunity to RSV through recruitment of antiviral monocytes. J Exp Med 2015; 212:699–714.

153. Elavazhagan S, Fatehchand K, Santhanam V, Fang H, Ren L, Gautam S, Reader B, Mo X, Cheney C, Briercheck E, et al. Granzyme B Expression Is Enhanced in Human Monocytes by TLR8 Agonists and Contributes to Antibody-Dependent Cellular Cytotoxicity. J Immunol 2015; 194:2786–95.

154. Williams MA, Newland AC, Kelsey SM. Monocyte-mediated killing of human leukaemia is enhanced by administration of granulocyte-macrophage colony stimulating factor following chemotherapy. Br J Haematol 1997; 98:960–8.

155. Griffith TS, Wiley SR, Kubin MZ, Sedger LM, Maliszewski CR, Fanger NA. Monocyte-mediated Tumoricidal Activity via the Tumor Necrosis Factor–related Cytokine, TRAIL. J Exp Med 1999; 189:1343–54.

156. Washburn B, Weigand MA, Grosse-Wilde A, Janke M, Stahl H, Rieser E, Sprick MR, Schirrmacher V, Walczak H. TNF-Related Apoptosis-Inducing Ligand Mediates Tumoricidal Activity of Human Monocytes Stimulated by Newcastle Disease Virus. J Immunol 2003; 170:1814–21.

157. Deng Z, Yan H, Hu J, Zhang S, Peng P, Liu Q, Guo D. Hepatitis C Virus Sensitizes Host Cells to TRAIL-Induced Apoptosis by Up-Regulating DR4 and DR5 via a MEK1-Dependent Pathway. PLoS ONE 2012; 7:e37700.

158. Lan L, Gorke S, Rau SJ, Zeisel MB, Hildt E, Himmelsbach K, Carvajal-Yepes M, Huber R, Wakita T, Schmitt-Graeff A, et al. Hepatitis C Virus Infection Sensitizes Human Hepatocytes to TRAIL-Induced Apoptosis in a Caspase 9-Dependent Manner. J Immunol 2008; 181:4926–35.

159. Volkmann X, Fischer U, Bahr MJ, Ott M, Lehner F, MacFarlane M, Cohen GM, Manns MP, Schulze-Osthoff K, Bantel H. Increased hepatotoxicity of tumor necrosis factor-related apoptosis-inducing ligand in diseased human liver. Hepatology 2007; 46:1498–508.

160. Mundt B, Kühnel F, Zender L, Paul Y, Tillmann H, Trautwein C, Manns MP, Kubicka S. Involvement of TRAIL and its receptors in viral hepatitis. FASEB J 2003; 17:94–6.

161. Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, Hillan K, Totpal K, DeForge L, Schow P, et al. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat Med 2001; 7:383–5.

162. Jang JY, Kim S-J, Cho EK, Jeong SW, Park EJ, Lee WC, Lee SH, Kim SG, Kim YS, Kim HS, et al. TRAIL enhances apoptosis of human hepatocellular carcinoma cells sensitized by hepatitis C virus infection: therapeutic implications. PloS One 2014; 9:e98171.

163. Kumar S. Caspase function in programmed cell death. Cell Death Differ 2007; 14:32–43.

164. Armstrong JS. The role of the mitochondrial permeability transition in cell death. Mitochondrion 2006; 6:225–34.

165. Seth RB, Sun L, Ea C-K, Chen ZJ. Identification and Characterization of MAVS, a Mitochondrial Antiviral Signaling Protein that Activates NF-κB and IRF3. Cell 2005; 122:669–82.

166. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. Front Immunol 2015; 6:257.

167. Décembre E, Assil S, Hillaire MLB, Dejnirattisai W, Mongkolsapaya J, Screaton GR, Davidson AD, Dreux M. Sensing of Immature Particles Produced by Dengue Virus Infected Cells Induces an Antiviral Response by Plasmacytoid Dendritic Cells. PLOS Pathog 2014; 10:e1004434.

168. Feng Z, Li Y, McKnight KL, Hensley L, Lanford RE, Walker CM, Lemon SM. Human pDCs preferentially sense enveloped hepatitis A virions. J Clin Invest 2015; 125:169–76.

169. Rimbach K, Kaiser S, Helm M, Dalpke AH, Eigenbrod T. 2'-O-Methylation within Bacterial RNA Acts as Suppressor of TLR7/TLR8 Activation in Human Innate Immune Cells. J Innate Immun 2015; 7:482–93.

170. Hajarizadeh B, Grebely J, Dore GJ. Epidemiology and natural history of HCV infection. Nat Rev Gastroenterol Hepatol 2013; 10:553–62.

171. Larrubia JR, Moreno-Cubero E, Lokhande MU, García-Garzón S, Lázaro A, Miquel J, Perna C, Sanz-de-Villalobos E. Adaptive immune response during hepatitis C virus infection. World J Gastroenterol WJG 2014; 20:3418–30.

172. Clarke SRm, Barnden M, Kurts C, Carbone FR, Miller JF, Heath WR. Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. Immunol Cell Biol 2000; 78:110–7.

7 Publication & Conferences

7.1 Manuscript submitted for publication

Klöss V, Grünvogel O, Wabnitz G, Eigenbrod T, Lohmann V, Dalpke A. Interaction and mutual activation of different innate immune cells is necessary to kill and clear Hepatitis C virus infected cells (submitted).
(The present thesis was based on the above mentioned submitted publication.)

7.2 Conferences

Innate Immunity in Host-Pathogen Interactions, EMBL Heidelberg, Germany, June 26-29, 2016
Poster presentation:
Klöss V, Grünvogel O, Wabnitz G, Eigenbrod T, Lohmann V, Dalpke A. Interaction of innate immune cells leads to specific killing of Hepatitis C subgenomic replicon cells

4th European Congress of Immunology, Vienna, Austria, September 6-9, 2015
Poster presentation:
Klöss V, Grünvogel O, Wabnitz G, Eigenbrod T, Lohmann V, Dalpke A. Trans-activation of innate immune cells by Hepatitis C subgenomic replicon cells

19th Symposium Infection and Immunity Burg Rothenfels, Rothenfels, Germany, March 19-21, 2015
Oral presentation:
Klöss V, Grünvogel O, , Eigenbrod T, Lohmann V, Dalpke A. Trans-activation of innate immune cells by Hepatitis C subgenomic replicon cells

Acknowledgements

Special thanks to Prof. Alexander Dalpke for giving me the chance to do my PhD thesis in his lab and for great discussions, advice and new ideas. I admire your calmness, your thoughtfulness and your positive attitude, which I benefited a lot from.

I would like to thank Prof. Michael Lanzer for agreeing to chair my examination committee and Prof. Dirk Grimm for agreeing to participate in the examination committee.

I also want to thank Dr. Tatjana Eigenbrod for supervision and our collaboration partners PD Dr. Volker Lohmann, Dr. Katharina Esser-Nobis and Oliver Grünvogel for helpful discussions and their great support in this project. Special thanks to PD Dr. Volker Lohmann for taking the time to participate in my thesis advisory committee and for refereeing my thesis. My thank also goes to PD. Dr. Guido Wabnitz for the nice collaboration and for being part of my thesis advisory committee.

Of course I would like to thank everyone else in the AG Dalpke for the nice working atmosphere and for always being supportive. Thank you Jana, Vedrana, Isabel, Sebastien, Anna, Michael, Suzan, Selina, Sabrina, Denis, Lauren and Lisa.

Moreover I want to thank all the students that worked with me on this project in lab rotations and their master thesis. Thanks to Aline, Henrike, Ann-Christin and Steffi.

I want to thank HBIGS for providing me with the MSc/PhD fellowship, for their support in this time and the great courses they offer.

Last but not least I want to thank my family and Lena who, most of the time, were able to make me forget the frustration of lab work.