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Of Natural Killer Cells *and* Hepatitis C Virus

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Declaration

I hereby declare that the thesis at hand has been written independently and with no other sources and aids than quoted.

The work described in this thesis was performed at the German Cancer Research Center (DKFZ), Heidelberg, Germany from April 2013 to October 2016 in the research group 'Innate Immunity' under the supervision of PD Dr. Adelheid Cerwenka.

Julia Pollmann

Unser Leben ist das, wozu unser Denken es macht.

- Marc Aurel -

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1 Zusammenfassung

Natürliche Killer (NK) Zellen sind wichtige Effektorzellen in Hepatits C Virus (HCV) Infektionen. HCV ist ein Virus mit dem ca. 2.5% der Weltbevölkerung chronisch infiziert ist und der einen wesentlichen Anteil zu Lebererkrankungen und Leberkrebs beiträgt. Die genauen Mechanismen durch welche NK Zellen von HCV aktiviert werden sind jedoch noch unklar.

Mit Hilfe des HCV Replikon Zellkultursystems konnten wir nachweisen, dass nach Co-Kultur von Replikon-tragenden Hepatozyten und mononukleären Zellen des peripheren Blutes (PBMCs) eine Subpopulation von NK Zellen die Expression der hoch-affinen IL-2 Rezeptorkette CD25 steigerte, verstärkt proliferiert hatte und mehr IFN-γ produzierte. Die Aktivierung von NK Zellen war abhängig von IL-2, welches sehr wahrscheinlich von T Zellen produziert wurde, und von Zell-Zell-Kontakt vermittelten Signalen von Monozyten. Diese zeigten in Co-Kulturen mit HCV Replikon eine erhöhte Expression von OX40L, einem Mitglied der Tumornekrosefaktor-Familie. Gleichzeitig war der Rezeptor OX40 auf NK Zellen stärker exprimiert. Blockade von OX40L und Depletion von Monozyten verhinderten die Virus-bedingte Aktivierung und gesteigerten Effektorfunktionen von NK Zellen.

Zusammenfassend zeigen unsere Daten einen neuen Mechanismus der Monozyten- vermittelten Aktivierung von NK Zellen gegen virusinfizierte Zellen und die Wichtigkeit der OX40/ OX40L Achse mit einer potentiellen Relevanz für eine therapeutische Intervention durch z.B. agonistische Antikörper gegen OX40, welche bereits in der Krebstherapie getestet werden.

2 Summary

Natural Killer (NK) cells are important effector cells in Hepatitis C Virus (HCV) infection, a virus that chronically infects around 2.5% of the world population and is a major cause of liver disease and hepatocellular carcinoma. The exact mechanisms, however, through which NK cells are activated in response to HCV remain elusive.

Using the well-established HCV replicon cell-culture model we show that after co-culture of HCV replicon-carrying hepatocytes with peripheral blood mononuclear cells (PBMCs), NK cells increase expression of the high-affinity IL-2 receptor chain CD25, proliferate rapidly and produce IFN-γ. Activation of NK cells was dependent on IL-2, most likely produced by T cells and on cell-cell contact mediated signals from monocytes. Monocytes from replicon-carrying co-cultures showed increased expression of OX40L, a member of the tumor necrosis factor family and concurrently its receptor OX40 was increased on NK cells. Blocking of OX40L in those co-cultures, as well as depletion of $CD14^+$ monocytes abrogated the virus-induced activation and effector functions of NK cells.

Together, our data reveals a novel mechanism of monocyte mediated NK cell activation against virus-infected cells involving the OX40/OX40L axis with potential relevance for therapeutic intervention by e.g. agonistic antibodies against OX40, which are already tested in cancer therapy.

3 Introduction

3.1 The human immune system

Every living organism - from the smallest bacteria through plants and animals to humans - is constantly exposed to pathogens. Throughout evolution, in ever more complex ways, all of these species have developed manifold strategies to protect themselves from harm. Emerged from this has the human immune system - the most complex immune system we know.

As manifold as the pathogens and their sites of infection are, as manifold are the defence mechanism of the body. The easiest and probably most effective defence are the physical, chemical and biological surface barriers. In humans, already an intact epithelial surface wards off many bacteria, protozoa, worms, fungi or viruses. Those areas not covered by epithelial, such as the gastro-intestinal tract and the respiratory system have strategies such as the low pH in the stomach that kills many pathogens that are ingested with food. The lungs are covered with mucus that traps bacteria and lined with cilia, that move the mucus out. Furthermore mucus, saliva, tears and other body fluid contain anti-microbial substances, such as the β-defensin peptides or the enzyme lysozyme, both of which can destroy the integrity of the invaders outer membrane.

However, pathogens have evolved effective mechanisms to counteract the body's defence and are therefore able to enter and infect the blood and organs of the body.

To efficiently guard the body, the immune system is spatially distributed, with immune cells and other immune components being present in virtually every organ and tissue. Furthermore many organs have developed tissue-specific immunity.

The function of the immune system is based on the principle of self vs. non-self discrimination. This discrimination is achieved via a plethora of receptors which can recognise

foreign, non-self structures on pathogens, but also altered-self structures, such as mutations in cancer cells. One type of receptors are the so-called pattern recognition receptors (PRRs). They are invariable, germ-line encoded receptors that recognise conserved structures on pathogens, called pathogen-associated molecular patterns (PAMPs). The second type of receptors are the highly variable T cell receptors (TCRs) and B cell receptors (BCRs), where each receptor recognises only one specific structure, its antigen. Based, amongst other criteria, on the two types of receptors the immune system can be divided into two branches - the innate and the adaptive immune system, with each of these branches having a humoral and a cellular part (*1*).

3.1.1 The adaptive immune system

One of the key features of the adaptive immune system is immunological memory which is conferred by the highly-diverse antigen-specific receptors present on T and B cells. Upon encountering their cognate antigen, T or B cells carrying the specific receptor undergo clonal expansion, maturation and subsequently a pool of memory cells is formed. These memory cells can 'remember' the previous antigen-encounter and, upon a secondary infection with the same pathogen, mount a faster and stronger secondary response. Immunological memory is the basis of a successful vaccination.

Although the adaptive immune system, as we understand it, only developed in jawed vertebrates, it is now known that already bacteria can remember and more effectively fight a secondary infection from e.g. bacteriophages by using the CRISPR-Cas system.

The cells of the human adaptive immune system are T and B cells. T and B lymphocytes, like all leukocytes, are produced in the bone marrow from hematopoietic stem cells and T cells are further differentiated in the thymus. Each T cell expresses a unique TCR which is composed of an α- and a β-chain and produced in a process termed somatic V(D)J recombination. recombination-activating gene (RAG)1/2 recombinases, randomly combine genetic elements from the TCR locus to form both chains. The entire TCR rearrangement process can produce 10^{14}-10^{18} TCRs with different specificity. T cells with unsuccessfully recombined chains, non-reactive and self-reactive TCRs are afterwards depleted in the thymus. The BCR is produced in a similar process and composed of 2 heavy transmembrane chains and two light chains. The BCR binds to antigens in their native configuration and can therefore detect extracellular bacteria and neutralise toxins. B cells can shed their BCRs into the plasma, where they are

called Immunoglobulins (Ig) or antibodies. There are five different Ig isoforms: IgA, IgD, IgE, IgG and IgM each with different functions and localisation, which make up the humoral part of the adaptive immune system.

The TCR recognises its cognate antigen only when it is presented in major histocompatibility complexs (MHCs). $CD8^+$ cytotoxic T cells recognise peptides presented in MHC-I, which is expressed on every nucleated cell. Activation of CD8 T cells via the TCR and co-stimulatory molecules results in killing of the target cell. $CD4^+$ helper T cells recognise peptides presented in MHC-II molecules, which are only present on specific antigen-presenting cells (APCs), such as macrophages, dendritic cells (DCs) or B cells. Activated CD4 T cells help for example macrophages to more efficiently phagocytose or B cells to produce antibodies.

Once a lymphocyte becomes activated it undergoes clonal proliferation to increase the number of cells specific for the pathogen (*1*) (*2*).

3.1.2 The innate immune system

The innate immune system is evolutionary much older than the adaptive immune system and found in different complexities in nearly all life forms.

In humans, the innate immune system is composed of the already mentioned surface barriers, a humoral part, primarily consisting of the complement system and a cellular part, which includes phagocytic cells (macrophages/ monocytes, dendritic cells and neutrophils), mast cells, eosinophils, basophils and innate lymphoid cells (ILCs), to which Natural killer (NK) cells belong.

The innate immune system is the first line of defense against invading pathogens, as it can act fast, without the need of prior activation, in a generic, antigen-independent manner. The innate immune system is activated via its germ-line encoded invariant PRRs that recognise e.g. viral and bacterial proteins and RNA/DNA. The most prominent class of PRRs are the Toll-like receptors (TLRs). Table 3.1 summarizes the human TLRs, their main ligands and cellular location. Monocytes/ Macrophages express almost all TLRs summarized in table 3.1, but many of the TLRs are also expressed on neutrophils, DCs, mast cells and also on B cells. More recently mRNA expression of different TLRs was observed in human NK cells. Data are still controversial, but most studies observed expression of TLR1, 2, 3, 5 and 6. TLR2 could also be detected on protein level by flow cytometry after cytokine activation. When stimulated with different

PAMPs or TLR agonists and cytokines such as IL-2, IL-12, IL-15 or IL-18, isolated NK cells were able to produce IFN-γ. In general, it has been observed that the activation of NK cells by PAMP seems to depend on the presence of activating cytokines produced by accessory cells (*3*).

All TLRs, except TLR3, use MyD88 as an adaptor protein, which leads to the activation of the immunological master transcription factor nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) and the activation of mitogen-activated protein (MAP) Kinases. TLR3 uses the adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF), which also activates NF-κB, but furthermore activates Interferon regulatory factor (IRF)3, leading to the production of type I Interferons (IFNs) (*1*) (*2*).

Table 3.1: Toll-like receptors and their ligands

Receptor	Ligand	Cellular Location
TLR 1	triacyl lipopeptides	plasma membrane
TLR 2	lipopeptides, glycopeptides	plasma membrane
TLR 3	double-stranded RNA, poly I:C	endosome
TLR 4	lipopolysaccharide (LPS)	plasma membrane
TLR 5	flagellin, profilin	plasma membrane
TLR 6	diacyl lipopeptides	plasma membrane
TLR 7	single-stranded RNA	endosome
TLR 8	single-stranded RNA	endosome
TLR 9	unmethylated CpG DNA	endosome
TLR 10	unknown	unknown

While the TLRs are restricted to the plasma membrane and the endo-/lysosome, other PRRs are present in the cytoplasm, namely the Rig-like receptors (RLRs), NOD-like receptors (NLRs) and Aim2-like receptors (ALRs). For intracellular viral recognition, the family of RLRs, consisting of retinoic acid-inducible gene-I (RIG-I), melanoma differentiation gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) are of

great importance. RIG-I recognises short, uncapped RNA and MDA5 double-stranded RNA. In general, they recognise RNA structures, which are common to viral RNA or viral replication intermediates, such as long double-stranded RNA or the poly-uridine region in the HCV genome. RIG-I and MDA5 recruit the adaptor protein mitochondrial antiviral signaling protein (MAVS) via its CARD-domain. This leads to the activation of NF-κB, IRF3 and IRF7 and thereby to the expression of pro-inflammatory cytokines such as IFNs and the transcription of IFN-stimulated genes (ISG), putting the cell into an anti-viral state (*4*).
In accordance with their function as intracellular pathogen sensors, expression of the RLRs, NLRs and ALRs is not restricted to the immune system, but found in various cell types throughout the body. E.g. RIG-I is highly expressed in the liver and muscle (*5*).

Monocytes and Macrophages

Monocytes are produced in the bone marrow from the common myeloid progenitor, which gives rise to the granulocyte-monocyte precursor, which eventually produces monocytes. They compose around 10% of leukocytes in the blood. Once they enter a tissue they differentiate into macrophages. Depending on their location macrophages have different names and organ-specific functions. In the liver for example, they are called Kupffer cells, in the brain microglia and in the lung alveolar macrophages.

Monocytes and macrophages are professional phagocytic cells and crucial players in the innate immune defence. They can engulf pathogens or cancer cells, but also clean up cellular debris and apoptotic cells, which is of importance in tissue repair and remodelling and during development.

Besides their direct anti-pathogenic functions, they play an extremely important role in shaping the overall immune response to a pathogen. Firstly, monocytes can produce a wide array of pro- and anti-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and Interleukin-10 (IL-10) and chemokines after stimulation with e.g. TLR-ligands, which regulate and attract other immune cells. Secondly, they are professional APCs. After TLR-activation and phagocytosis of a pathogen they process pathogenic antigens and present them in MHC-II molecules to CD4 T cells that carry the TCR specific for this particular antigen to activate them. This way monocyte form a bridge between the innate and adaptive immune system (*1*) (*2*).

Macrophages can be classified into three major subtypes according to their surface expression of CD14, a co-receptor for TLR4, and FcγRIII CD16. $CD14^{++}$ $CD16^{-}$ are classical monoytes, $CD14^{++}$ $CD16^{+}$ are intermediate monocytes and $CD14^{+}$ $CD16^{++}$ are the non-classical monocytes (*6*).

Another classification is made according to their way of activation and the thereof resulting function into classically activated M1 macrophages and alternatively activated M2 macrophages. M1 type macrophages have a pro-inflammatory phenotype, which promotes killing of intracellular pathogens and tumour resistance. They develop after stimulation with e.g. Interferon-gamma (IFN-γ), LPS, granulocyte macrophage colony-stimulating factor (GM-CSF) or after intracellular TLR activation by viruses. Functionally they upregulate inducible nitric oxide synthase (iNOS) to increase production of the free radical nitric oxide (NO), increase antigen-presentation and T cell co-stimulation via high expression of MHC-II and CD86. Furthermore they produce IL-12, IL-1, IL-6 and TNF-α. M2 macrophages on the other hand have diverse functions in allergy, immune responses to parasites and in the promotion of tissue repair. They are amongst others stimulated by IL-4, IL-10 and IL-13 and produce cytokines such as IL-10 or transforming growth factor-beta (TGF-β) (*7*).

3.2 Natural Killer Cells

NK cells have been identified in mice in 1975, when researchers observed a 'natural', spontaneous cytotoxicity against certain tumor cell lines from splenic cells (*8*)(*9*). They have been termed 'natural' killer cells, because, unlike e.g. T cells, they did not require sensitisation or priming prior to exerting their effector functions. The fact that these killer cells with natural cytotoxicity were specifically recognising targets that had low or no MHC-I expression, which is a sign of transformation or infection of a cell, led, more than 10 years after their first description, to the discovery of their mode-of-activation, when Klas Kärre and Rolf Kiessling proposed the 'missing-self' hypothesis (*10*). This states that NK cells do not only, like other immune cells, become activated by the presence of non-self structures, but can also recognise infected/transformed cells via the absence of self-structures, in this case MHC-I. Today we know that NK cells express a great number of receptors, primarily belonging the killer-cell immunoglobulin-like receptor (KIR) family of receptors, that can bind MHC-I molecules and inhibit NK cell activation. Together with signals derived from activating receptors, that mainly recognise stress-induced ligands, the activation status of an NK cells is defined. Activation

of NK cells not only leads to target cell killing, in addition NK cells are also potent cytokine producers, the key cytokine being IFN-γ, through which they play an important part in orchestrating the overall immune response.

NK cell receptors are germ-line encoded and do not undergo genetic recombination by RAG1/2. Therefore NK cells are classified as innate immune cells, although they share a common progenitor cell with T and B lymphocytes and many functional and transcriptional similarities to CD8 T cells. Of note, a recent publication reported a role for the RAG recombinase in NK cell development and cellular fitness (*11*). Since a few years, we now appreciate that NK cells are not the only innate cells that are derived from the common lymphoid progenitor cell, but do, in fact, belong to a whole group of ILCs (*1*) (*2*).

3.2.1 NK cell definition, development and subsets

Definition

NK cell are cytotoxic lymphocytes that constitute around 5% of leukocytes or 10% of lymphocytes in the peripheral blood. Phenotypically NK cells are clasically defined as $CD3^-$ (non-T) cells that express CD56, an isoform of neural cell adhesion molecule (NCAM). CD3 has to be excluded because CD56 is also expressed on $CD3^+$ NKT cells. More recently, NKp46 has been proposed as a more exclusive and general NK cell marker, as NKp46, unlike CD56, is also expressed on mouse NK cells (*12*). Furthermore the occurence of $CD56^-$ NK cells has been observed in certain viral infections, which also encourages the use of NKp46 as an NK cell marker (*13*). However, expression of NKp46 can also be found under certain conditions on subsets of T cells (*14*) and it is also expressed on ILCs3 and ILC1 subsets (*15*). As long as no unique NK cell marker has been identified, CD56 and NKp46 will be used, with the above mentioned reservations in mind.

Development

NK cells develop in the bone marrow from Lin^- $CD34^+$ hematopoietic stem cells. However, this may not be the only site of NK cell development, as NK cell precursors are also present in the circulation and $CD34^+$ NK precursors are enriched in other lymphoid and non-lymphoid organs, such as the thymus, lymph nodes, the gastrointestinal tract

or the liver. (*16*). Compared to mouse NK cell development, rather little is known about NK precursors in humans and much of the data comes from *ex vivo* differentiation experiments.

In humans it is thought that NK cells and ILCs develop from the same common lymphoid progenitor as T and B cells. During the commitment to the NK cell lineage, the aquisition of CD122, the IL-15 receptor β chain, marks an important step. IL-15 is crucial for NK cell homeostasis and promotes survival and proliferation of NK cells. *In vitro* IL-15 is also capable to differentiate $CD34^+$ hematopoietic progenitor cells into NK cells (*16*). Immature NK cells do not yet express inhibitory KIRs and CD94/NKG2A on their surface, making them potentially auto-reactive, but it is commonly thought that they do not yet possess effector functions. During further maturation NK cells acquire KIR, CD94/NKG2A, CD56, NKp46, CD16 and Natural Killer group 2, member D (NKG2D).

NK cell education

The acquisition of KIRs is a stochastic process, which leaves some NK cells expressing no KIRs at all, others expressing one or even multiple different KIRs. Studies have shown, that mature NK cells that lack expression of inhibitory receptors are in fact, not hyper- but hyporesponsive. Furthermore it was shown that the signaling motifs of inhibitory receptors (Immunoreceptor tyrosine-based inhibitory motifs (ITIMs)) are crucial to achieve proper responsiveness of NK cells. Out of this and many other observations, the concept of NK cell education has developed (*17*). NK cells are activated by the sensing of 'missing-self' and according to the NK cell education concept, in order to do so, NK cells have to be educated to the normal MHC-I environment of the host. During NK cell education, those NK cells that express many inhibitory receptors that have a matching MHC-I allele or express inhibitory receptors with high affinity for their respective ligand become 'licensed' to excert greater effector functions after stimulation (*18*).

Besides KIRs, the CD94/NKG2A heterodimeric receptor, which recognises the non-classical MHC-I molecule HLA-E, also plays a role in NK cell licensing. CD94/NKG2A is presumably of even greater importance in the early stages of NK cell maturation. As of now, the prevailing opinion is that committed NK cells first acquire a $CD56^{bright}$ phenotype and during maturation reduce CD56 expression. Almost all $CD56^{bright}$ NK

cells are expressing CD94/NKG2A, but little or no KIRs. This balance is reversed during the maturation into $CD56^{dim}$ NK cells. Only about half of the $CD56^{dim}$ NK cell subset expresses CD94/NKG2A (*17*).
Of note, this does not mean that uneducated NK cells cannot become activated and exert effector functions. In fact, under certain conditions, they might even perform better than their educated counterparts. For example, they have been shown to better react to MHC-I^+ cancer cells and be superior in the clearance of murine cytomegalovirus (MCMV) (*17*).

NK cell subsets

The two major NK cell subsets are, as already mentioned, the $CD56^{dim}$ and the $CD56^{bright}$ subset, which are though to represent two phases in a linear NK maturation model. The more immature $CD56^{bright}$ subset, which comprises around 10% of the blood NK cells under homoeostasis, but it the prevalent NK subset in secondary lymphoid organs, has a high potential to produce cytokines, but is less cytotoxic than the $CD56^{dim}$ NK cells. The $CD56^{dim}$ subset has a high cytotoxic potential and due to the co-expression of CD16 (which lacks in $CD56^{bright}$ cells) has the potential to exert antibody-dependent cellular cytotoxicity (ADCC). CD16 recognises the constant region of the heavy chain on an antibody, the Fc-part, bound to its antigen and thereby triggers NK cell degranulation. Of note, this classification is somewhat simplified and under the right conditions, both subsets can be cytotoxic and cytokine expressing.

3.2.2 NK cell receptors, ligands and their activation

NK cells have a plethora of activating and inhibitory receptors on their surface, as well as cytokine, chemokine and adhesion receptors. They can be classified into several subgroups, according to their structure, function and/or ligands. Most activating receptors signal via immunoreceptor tyrosine-based activation motifs (ITAMs), most inhibitory via ITIMs in their cytoplasmic tail or associated receptors/ adaptors. ITIM activation activates phosphatases such as SHP-1/2 or SHIP which dephosphorylate and thereby inhibit the function of further downstream effectors, while ITAM activation stimulates phosphorylation, e.g.. via phosphatidylinositol-3 kinase (PI3K), of those downstream effectors. The integration of all signals from inhibitory and activating receptors finally decides if an NK cell is activated or not.

Natural Cytotoxicity Receptors

One major group of activating NK cell receptors are the natural cytotoxicity receptors (NCRs). Members of this family of receptors include NKp46, NKp30, NKp44 and NKp80. They all contain one or two immunoglobulin-like domains and couple to intracellular adaptor molecules expressing ITAMs. Besides that they do not share sequence homology, but are rather grouped together because of their potential to activate NK cells (*19*).
NKp46 is constitutively expressed on all NK cells and NKp30 is found on all mature NK cells (*20*). NKp44 is not expressed in steady-state, but inducible after activation (*21*). For many of the NCRs, the ligands are poorly defined, especially the cellular ligands. The first ligands that were discribed for NKp46, NKp44 and NKp30 were, actually, viral proteins. Hemagglutinin neuraminidases (HN) and haemagglutinins (HAs) of different viruses, such as influenza, Sendai virus, vaccina virus or Newcastle disease virus have been shown to bind to some of these NCRs (*19*). Furthermore human cytomegalovirus (HCMV) tegument protein pp65 can bind to NKp30 (*22*). Cellular ligands include proliferating cell nuclear antigen (PCNA) and NKp44L for NKp44 and B7-H6 for NKp30, which is one of the few extracellular ligands described on the cell surface of tumour cells (*23*)(*24*).

Natural Killer Group 2 Receptors

The class of Natural killer group 2 receptors on NK cells includes activating as well as inhibitory receptors. NKG2C, NKG2E and NKG2D are the activating forms belonging to this C-type lectin-like family of receptors and NKG2A is inhibitory. NKG2D is among the best studied NK receptors. It is expressed on almost all NK cells, but also on T cells, where is acts mainly as an co-stimulatory molecule. It signals via the adaptor molecule DAP-10 and recognises the stress-induced MHC-I homologues MHC class I polypeptide-related sequence A (MIC-A) and MHC class I polypeptide-related sequence B (MIC-B) and UL16 binding protein (ULBP)1-6 (*25*). Other members of the NKG2 family have the non-classical HLA-E molecule as ligand.

Killer-cell Immunoglobulin-like Receptors

The KIRs are a highly polymorphic group of receptors binding to the equally polymorphic MHC-I molecules on target cells. Most of the known KIRs are inhibitory, but some, lesser defined KIRs, are also activating NK cells. The KIRs are involved in the already mentioned 'missing-self' activation of NK cells and in the licensing/education process during NK development.

TNF-Receptor Superfamily

While the NCRs, NKG2 receptors and KIRs are receptors that are primarily expressed on NK cells, the expression of the members of the tumor necrosis factor receptor superfamily (TNFRSF) and their ligands, the tumor necrosis factor superfamily (TNFSF) are widely expressed in lymphoid and non-lymphoid tissues and play a major role in adaptive and innate immune responses against pathogens and also in cancer by facilitating death of an infected/transformed cell, but also by modulating immune cells. NK cells are known to express several TNFRSFs and TNFSF members.
This family of receptors is further subdivided into two subgroups: the death domain-containing receptors (e.g. Fas, TRAIL-receptors (TRAIL-Rs), tumor necrosis factor receptor (TNFR)1) whose activation induces caspase activation and apoptosis and the TNFR-associated factor (TRAF)-binding receptors (eg. OX40, CD40, HVEM, GITR, 4-1BB, CD27, CD30) which activate transcription factors like NF-κB and are therefore involved in cell activation, survial and differentiation (*26*).

TNFSF members are often induced after NK cell activation and serve as effector molecules that induce cell death in a target cell. The TNFSF members expressed by NK cells include the prototypic TNF-α as well as FasL and TNF-related apoptosis inducing ligand (TRAIL), which lead to the induction of apotosis in the target cell. Furthermore NK cells express HVEM ligands LIGHT, Lymphotoxin-α/β, BTLA and CD160, whose interaction with HVEM is rather complicated, as it can be inhibitory or activating depending on the ligands (LIGHT and Lymphotoxin binding is thought to promote inflammation and BTLA and CD160 is rather anti-inflammatory). And also because HVEM signalling can be bidirectional, as many of the ligands have signalling capacity themselves. NK cells express not only the ligands for HVEM, but also HVEM itself (*27*) (*28*).

Other TNFRSF members expressed by NK cells are GITR and 4-1BB. 4-1BB is almost absent and GITR only expressed at low levels on resting human NK cells, but both can be induced after e.g. cytokine activation. For both receptors activating as well as inhibitory functions have been described for NK cells, while on T cells they work as co-stimulatory molecules. 4-1BB and GITR agonistic antibodies are in clinical trials to boost T cell anti-cancer efficiency. In NK cells the context of GITR or 4-1BB stimulation may be important to determine the outcome of receptor engagement. Possible factors include the cytokine milieu, the nature of the cell expressing the ligand, or the form of the ligand (soluble vs. cell-bound) (*26*).

TNFSF-Receptor OX40 and its ligand OX40L OX40 (also known as CD134 or TNFRSF4) is best known as a co-stimulatory receptor on T cells, primarily on $CD4^+$ T cells, but can also be expressed on NK, NKT cells and neutrophils. Data on the function of OX40 on NK cells is sparse, but all studies indicate it to function as an activating receptor as well. On almost all cell types OX40 is not expressed in the naive state, but has to be induced by activation. However, reports show constitutive expression on CD4 T follicular helper cells (*29*).

In T cells OX40 is induced after TCR and CD28 or CD40 engagement in a sequential manner, making it a late co-stimulatory molecule. The exact timing and duration of OX40 induction varies drastically between different experimental conditions and cytokine stimulation (eg. IL-1, IL-2, IL-4 or TNF) can also prolong and induce the expression of OX40 (*30*) (*31*).

OX40 can act as an independent receptor, like other TNFRs super family members, by recruiting several TRAF proteins and activating NF-κB. This results in enhanced cell survival. Furthermore, in collaboration with TCR signalling, it can activate both, NF-κB and the PI3K-Akt pathway, which leads to the stabilization of high levels of anti-apoptotic Bcl-2 family members (enhances survival), the induction of several molecules involved in cell cyle progression, such as survivin and cyclin A (enhances proliferation) and the production of cytokines (*30*). One cytokine that is produced by effector CD4 T cells after OX40 ligation is IL-2. Simultaneously expression of the IL-2Rα chain (CD25) is upregulated. Enhanced IL-2 signalling then promotes IFN-γ production of T cells in response to cytokines such as IL-12 or IL-18. Other effects of OX40 signaling on CD4 T cells include upregulation of IL-12Rβ2 chain and downregulation of anti-inflammatory mediators such as CTLA-4 and IL-10 (*30*).

Initial studies performed in OX40 knock-out mice showed that besides impaired survival, proliferation and cytokine production, also memory formation and maintenance was affected. Specific expression and importance of OX40 in memory T cells was then also confirmed in humans. When human CD4 memory T cells are rechallenged *in vitro* with viral, bacterial or autoantigens, they rapidly start to express OX40. In many reports OX40 expression coincides with CD25 upregulation. Co-expression of OX40 and CD25 on memory cells is now used in the clinics to determine whether a patient has encountered a specific pathogen before or is reactive to autoantigens (*32*). Keoshkerian et al. could show that HCV-specific CD4 memory cells upregulate OX40 and CD25 after stimulation with recombinant viral peptides or proteins and viral supernatants. This helps in identifiying patients with chronic and resolved HCV infection (*33*).

Studies on CD8 T cells, neutrophils, NK and NKT cells have yielded similar results concerning the effects of OX40 ligation in promoting survival, differentiation and enhanced effector functions, but other than for CD8 T cells, literature is very sparse. Indeed, to our knowledge, only one report showed staining for OX40 expression on NK cells and this report was done in mice (*34*). Other studies in humans only implied a function for OX40-OX40L interaction for NK cell function using blocking or agonistic antibodies (*35*) (*36*) (*37*).

No other interaction partners for both OX40 and OX40L (also known as CD134L and CD252) then with each other are known in humans and also in mice. Although interstingly, in cats OX40 serves as binding receptor for the feline immunodeficiency virus (*38*).

OX40L expression has been shown on APCs (B cells, macrophages, DCs), mast cells, activated T cells and NK cells. It can furthermore be expressed on non-immune cells such as bronchial smooth muscle cells, transformed cells or vascular endothelial cells (*31*). OX40L, like OX40 is only expressed after induction. In 2004 two studies showed that NK cells can induce the expression of OX40L after activation. Zingoni et al. demonstrate that only combined cytokine and NKG2D or CD16 triggering induces OX40L upregulation. In co-cultures with autologous $CD4^+$ T cells, OX40L expressing NK cells co-stimulated T cell proliferation and IL-2 or IFN-γ production (*39*) (*40*).

OX40/OX40L in the (pre)clinic Upregulation of OX40 and OX40L has been observed in a variety of autoimmune diseases, such as rheumatoid arthritis (*41*), colitis, muliple sclerosis and systemic lupus erythematosus. This sparked the development of OX40 and OX40L antagonistic antibodies to ameliorate diseases. In mouse models of autoimmune diseases treatment success with blocking/neutralizing antibodies showed both worsening and amelioration of disease, depending on the model and treament conditions. Although a human trial with humanized α-OX40L in asthma patients had no severe side effects, it was discontinued because of its inefficacy (*31*).

In sharp contrast to this, OX40 agonist treatment for cancer patients shows a huge potential for the advancement of immunotherapy.

It was shown that a large part of tumour-infiltrating CD4 T cells express OX40, establishing the prerequisit for using OX40 as a candidate for immunotherapy. Subsequently, in pre-clinical animal studies OX40 agonist or OX40L-Fc treatment alone or in combination with checkpoint blockade, radiation or cytokine treatment had a significant survival benefit in almost all of the many studies performed (*29*).

The first human trial was conducted in 2013 with a mouse monoclonal α-human OX40 agonist. Treatment with only one dose led to the regression of metastasis in almost half of the patients with advanced disease, while side-effects were acceptable and milder, when compared e.g. to α-CTLA4 checkpoint blockade. CD4 and CD8 T cells, but not Tregs were expanded and activated in the tumour. Unfortunately, patients developed anti-mouse antibodies and therefore the trial had to be stopped (*36*) (*29*). Several other trials are being conducted or planned at the moment, including combination trials using OX40 agonists and CTLA-4/ PD-1 blockade. Furthermore triple therapy including e.g. KIR-blockade, which is also being investigated in clinical trials at the moment, is envisaged for the near future.

Cytokine receptors

Cytokines and chemokines have an immense impact on all aspects of NK cell biology. They are critical during NK cell development, they impact maturation/ differentiation, migration/ homing, survival/ proliferation and effector functions. More recently, cytokine treatment of NK cells has even been shown to produce memory-like NK cells, a feature that has previously thought to be restricted to the adaptive immune system (*42*). The most important cytokine receptors for NK cells are described below.

The common γ-chain receptors NK cells depend heavily on a family of cytokines which use di- or trimeric receptors in which one chain is the common γ-chain (CD132). The common γ-chain cytokines include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. All receptors consist of a specific α-chain combined with the common γ-chain, only IL-2R and IL-15R are trimeric and furthermore include the IL-2Rβ (CD122). The common γ-chain is important for signalling in all receptors. It binds to Janus kinase (JAK)3 which activates downstream signal transducers and activators of transcriptions (STATs) proteins by phosphorylation. Activated STATs dimerise, translocate to the nucleus and activate transcription of target genes. Depending on the cytokine, different STATs are preferentially activated (IL-2, -7, -9 and -15 mostly activate STAT-5, IL-4 STAT-6 and IL-21 STAT-1 and STAT-3) (*43*). The activation of different STAT proteins by JAK3 is facilitated by the differential ability of inactive STAT proteins to bind to the specific receptor α-chains. This leads to cytokine specific functions and NK cell activation.

Not only NK cells, but in fact all lymphocytes (T, B and NK cells) depend on common γ-chain/ JAK3 signalling. In humans and mice with genetic defects in either of the genes, all lymphocytes are missing, causing the severe combined immune deficiency (SCID) syndrome.

IL-4, IL-7 and IL-9 seem to have no or only little function in regulating peripheral human NK cell responses. Of note, IL-7 might be important for the development of organ-specific NK cells and IL-7R and IL-9R signalling is indispensable for the development and survival of certain ILC populations (*44*). IL-21 has mainly co-stimulatory functions and acts in concert with other cytokines to accelerate NK development, increase survival and effector functions, such as cytotoxicity and ADCC (*45*).

IL-2 and IL-15 are the most important cytokines for NK cell survival and proliferation. Although their effects on NK cells is similar, their expression and importance for NK cells differs.

IL-15, in contrast to IL-2 is most important during homoeostatic conditions. It is indispensable for NK cell development and more potent than IL-2 in promoting NK cell survival. The importance for NK maintenance is also mirrored in the production of these cytokines. IL-15 is constitutively expressed by a variety of cell types of immune and non-immune origin (monocytes, DCs, epithelial cells, stromal cells), while IL-2 is primarily produced by CD4 T cells after TCR activation. However, IL-2 as well as IL-15 treatment can stimulate NK cell effector functions and proliferation.

Furthermore, although both receptors use the IL-2Rβ chain and the common γ-chain, their α-chains and ligand binding vary greatly. In fact, IL-15 binding is quite unique. While it can bind as a free cytokine to the trimeric receptor, most commonly it is shuttled to the cell surface of the IL-15 expressing cell, already bound to the IL15Rα-chain. IL15/IL15Rα is then trans-presented to another cell expressing the β and γ-chain (*46*).

IL-2 can already bind with intermediate affinity to the β and γ-chain, but affinity is greatly increased if the IL-2Rα-chain (CD25) is incorporated into the receptor.

CD25, the high-affinity IL-2R chain Expression of CD25 under normal conditions is absent on $CD56^{dim}$ NK cells and T cells and only low levels are expressed on $CD56^{bright}$ NK cells, but it is constitutively expressed on regulatory T cells (Tregs) (*47*). Because of this and the fact that IL-2 is involved in T cell activation induced cell death, CD25 and IL-2 signalling also partake in immune regulation.

Various stimuli were shown to induce its expression on NK cells. For example stimulation of activating NK cell receptors NKG2D, NKp30 and NKp46 with soluble MIC-A, α-NKp30 or α-NKp46 antibodies respectively, increased CD25 expression (*48*). Enhanced percentage of $CD25^{+}$ NK cells were also observed after Influenza vaccinations (*49*). Furthermore CD25 is expressed on cytokine-stimulated NK cells, i.e. after IL-12 + IL-15 + IL-18 treatment (*50*).

IL-12, IL-18 and the type I IFN receptors IL-12 signals via STAT-3, -4 and -5 and can induce NK cytotoxicity and IFN-γ and TNF-α production. Cytokine production is mainly facilitated via STAT-4 signalling. Although it can act alone, IL-12 synergises with other stimuli. For example IL-12 together with IL-2 or IL-15 trigger NK IFN-γ, TNF-α and GM-CSF production. IL-12 also enhances tumor cell recognition significantly (*51*) (*52*).

IL-18 does not signal through JAK-STAT, but has MyD88 and TRAF6 as adaptors to activate MAPK and NF-κB. IL-18R is, like the IL-12R constitutively expressed on resting NK cells. IL-18 stimulation leads to minor NK proliferation which can be drastically enhanced by IL-15 co-stimulation. Also IL-18 primed NK cells can produce more IFN-γ after subsequent IL-12 stimulation (*52*).

IFN-α and -β can act on NK cells via the induction of JAK1 and different STAT proteins. STAT-1 and STAT-2 activation lead to increased cytotoxicity through an upregulation of perforin and FasL, whereas STAT-4 activation increases IFN-γ secretion. Type I IFNs furthermore increase NK cell proliferation. The most potent producers of type I IFNs are plasmacytoid dendritic cells upon virus encounter (*51*).

3.2.3 NK cell effector functions

Cytotoxicity

NK cells have several modes through which they can induce death of a target cell. One is through the production of TNFSF members, e.g. TRAIL and FasL, that activate the death domain-containing TNFRs, which were discussed in chapter 3.2.2. The other mechanism is via the release of cytotoxic granules, which mainly contain perforin and the serine proteases granzyme A and B, as active agents. After engagement of a target cell, an immunological synapse forms and the cytotoxic granules are released in close proximity to the target cells. Perforin is then necessary to deliver the granzymes into the target cell cytoplasms, but the exact mechanisms are still unclear. Once inside the cytoplasm, granzymes can induce apoptosis via cleavage of pro-caspases, through the induction of pro-apoptotic proteins or via the cleavage of mitochondrial and nuclear proteins which eventually causes mitochondrial depolarisation and DNA fragmentation (*53*)(*54*).

Cytokine production

IFN-γ - the NK signature cytokine The key cytokine produced by NK cells is without a doubt the sole member of the type II IFNs: IFN-γ. IFN-γ production, in contrast to the members of the type I IFN family, IFN-α and -β, which can be produced by virtually any cell type upon viral infection, is restricted to immune cells. Besides NK cells, T cells and NKT cells are also potent IFN-γ producers. Induction of IFN-γ in these cell types is largely controlled by cytokines. After e.g. pathogen encounter macrophages produce IL-12, which, in synergy with IL-18, potently induces IFN-γ production by NK cells. In turn, IFN-γ is then one of the most important activating cytokines for macrophages and stimulates many of its functions (*55*). In contrast to its limited production the effects of IFN-γ are not limited to the immune system, as the IFN-γ receptor is nearly ubiquitously expressed (*56*).

Although type I and type II IFNs are produced by different cells and acting on different receptors, the intracellular signalling of type I and type II IFNs largely converge to activate ISGs. The IFN-γR, like many other cytokine receptors signals mainly via the JAK-STAT pathway. The cytoplasmic tail binds to JAK1 and JAK2 which then facilitate STAT-1 phosphorylation. Phospho-STAT-1 dimers can then activate or suppress transcription of IFN-γ stimulated genes that contain the gamma Interferon activation site (GAS) element. The effects of the ISGs are manifold and embedded in a complex interplay with other cytokine signals. Below are listed some of the actions of ISGs, but this list can be expanded greatly, e.g. by the anti-proliferative and pro-apoptotic effects on cancer cells.

IFN-γ in antigen-presentation ISGs induced by IFN-γ include many that stimulate antigen-presentation in MHC-I and MHC-II complexes. Transcription of alternative proteasome components is initiated, which forms the immunoprotease and produces quantitively more and qualitatively better peptides to be loaded. Furthermore, many proteins aiding antigen-loading are transcribed (e.g. transporter associated with antigen-processing (TAP) or tapasin). It also directly upregulates chains of the MHC-I and MHC-II complexes, even in cells that do not constitutively express MHC-II (*55*).

IFN-γ in anti-microbial functions Macrophages are important effector cells in the defence against bacteria and parasites. IFN-γ actives their direct phagocytic potential, increases FcγRI to induce ADCC and increases complement and complement receptor production. Also the production of microbicidal products, especially NO and reactive oxygen species (ROS) production, is stimulated, which acts directly on the pathogens. This is mediated via induction of the NADPH oxidase system and iNOS (*55*).

IFN-γ antiviral functions Together with the type I ISGs, the IFN-γ-stimulated genes can directly interact with all steps in the viral life cyle. One type II ISG is for example the PKR kinase that binds the transcription initiation factor eIF-2α, and thereby inhibits cellular as well as viral protein translation, as almost all viruses use the cellular transcription machinery (*55*). PKR activation also stimulates degradation of the inhibitor of NF-κB, which activates NF-κB (*56*). ISGs interfere with viral entry, virus translation and replication, the formation and release of viral particles through the induction of a plethora of proteins.

Other cytokines produced by NK cells Other pro-inflammatory cytokines that are produced by NK cells are TNF-α and GM-CSF, the latter of which is important for the cross-talk with monocytes/macrophages. To attract other immune cells to the site of inflammation NK cells can produces the chemokines MIP-1α, MIP-1β, RANTES and IL-8. NK cells can as well produce anti-inflammatory cytokines like IL-5, IL-10 and IL-13 (*57*).

3.2.4 NK cells in the liver

The liver has a quite specific immunological environment that has to balance tolerance and inflammation carefully. On the one hand, the liver is constantly exposed to antigens from bacteria and food that are drained into the liver from the gut via the portal vein, to which the liver has to remain tolerant. This is promoted via a constant production of the anti-inflammatory cytokines IL-10 and TGF-β. The liver is therefore considered a more immun privileged organ. But on the other hand, it has to effectively fight pathogenic threats. For the activation of potent NK cell responses in this context cross-talk with Kupffer cells, the liver resident macrophages is very important (*58*).

The immune cells are primarily located in the sinusoids of the liver which spread through the liver parenchyma, composed of hepatocytes. As the sinusoids are quite permeable, contact between blood, immune cells and hepatocytes is guaranteed. The sinusoid endothelial cells are separated from the hepatocytes via the space of Disse, a narrow cleft where hepatic stellate cells reside (*59*).

The composition of immune cells is enriched in innate immune cells and especially in NK cells. In fact, 30-40% of lymphocytes in the liver are NK cells, a number that is only surpassed in the uterus, where around 50% of lymphocytes are NK cells. In comparison, the percentage in blood is only 10-15%. Under pathological circumstances, the percentage of NK cells in the liver can even reach up to 80 or 90%.

There are two types of NK cells present in the liver: the liver-resident NK cells and the conventional NK cells, which are thought to only pass through the liver via the blood. The liver-resident NK cells, as the name already suggests, rarely if ever leave the liver. They are CD56^{bright} and express chemokine receptors for CCL3, CCL5 and CXCL16, chemokines which are produced by many cell types in the liver, including hepatocytes and sinusoidal endothelial cells. They are also, like their mouse counterparts, positive for the integrin CD49a and most likely depend on different transcription factors than

concentional NK cells (Tbet instead of Eomes). In fact, studies in mice have shown the presence of hematopoietic precursor cells in the liver, that could replenish the liver-resident NK cell pool directly within the organ. In humans further studies have to provide inside into the origin and exact nature of the liver-resident NK cells and whether they should be classified as a distinct ILC subset (*58*).

Functionally the liver-resident NK cells are potent producers of a many cytokines, including IFN-γ, TNF-α and GM-CSF and they were shown, similar to mouse liver NK cells, to possess a memory-like phenotype (*59*).

3.2.5 NK - monocyte/macrophage cross-talk

NK cells can directly recognise infected or transformed cells via their cell surface receptors and exert effector functions. However, the nature and strength of their effector functions, survival, trafficking and other functions are strongly influenced by the surrounding immunological milieu. Especially in the early phases of an immune response the interaction with myeloid and dendritic cells is of great importance. Monocytes and macrophages are, like NK cells, among the first cell types present at a site of inflammation and they have the potential to affect NK cells via cytokines or cell-cell-contact dependent mechanisms.

The interaction of monocytes/macrophages and NK cells has been shown to stimulate CD69 expression, a marker of NK cell activation and NK cell effector functions in many viral, bacterial and parasitic infections (*60*). This interaction is facilitated via different pathways.

Monocytes which are strongly stimulated with LPS or infected themselves with eg. Mycobacterium tuberculosis upregulate NKG2D-Ligands (MIC-A/B and ULPBs) on their surface. This cell-to-cell contact dependent interaction preferentially leads to the killing of the monocytes to eliminate the pathogen in case they are directly infected and is thought to prevent sepsis in case of overactivated monocytes. Like NKG2D, interaction via NKp46 has also been primarily implicated in the direct killing of monocytes (*60*). NKp30-ligand B7-H6 could be induced on inflammatory monocytes by LPS or IL-1β stimulation and co-culture of B7-H6 expressing monocytes with autologous NK cells also resulted in higher NK degranulation. Of note, IFN-γ production was not addressed in this study (*61*).

However, low-dose LPS stimulation rather activates interaction via the 2B4-CD48 axis which leads to NK cell cytokine production, rather than cytotoxicity (*62*). Therefore, depending on the type and the strength of monocyte activation, different cell-to-cell contact dependent pathways can be activated leading to either NK cytotoxicity and killing of monocytes or to NK cell cytokine production.

Beside the cell-cell-contact dependent monocyte - NK cell interaction, chemokines and cytokines secreted by monocytes have a major impact on NK cell immunity.

After pathogen encounter monocytes and macrophages can produce pro-inflammatory cytokines such as IL-12 and IL-18, whose impact on NK cells is described in chapter 3.2.2 and 3.2.3. IL-12 can be produced e.g. after infection with human cytomegalovirus by inflammatory monocytes and was shown to play a role for the expansion of a subset of NK cells carrying the NKG2C receptor which is thought to have adaptive features (*63*).

Cytokine-production by Kupffer cells, the liver-resident macrophages was shown after *in vivo* stimulation of human Kupffer cells with TLR-2, -3 or -4 ligands. In all conditions, Kupffer cells produced pro-inflammatory IL-12, IL-18, TNF-α, but also the anti-inflammatory cytokine IL-10. TLR-2 stimulation was also able to induce IL-1β in Kupffer cells.

3.3 Hepatitis C Virus

Hepatitis C Virus was discovered in 1989, after years of intensive research to find the causative agent of the clinical non-A, non-B hepatitis (*64*).

HCV is a hepatotropic, enveloped virus. It is phylogenetically grouped into seven major genotypes. Each genotype (1-7) can be further subdivided into many subgenotypes. In western countries, the most prevalent subgenotypes are 1a, 1b, 2a, 2b, 3a, 4a and 6a. Furthermore, due to the high error-rate of the viral polymerase, in every individual many slightly different viral genome, so called quasispecies, are present (*65*).

3.3.1 Virology

Hepatitis C virus (HCV) is a single-stranded positive sense ss(+)RNA virus that belongs to the genus hepacivirus of the flaviviridae family. Other well-known human pathogens of the flaviviridae family are Dengue virus (DENV), West Nile Virus, Zikavirus and Yellow Fever Virus, all of which are members of the flavivirus genus.

HCV is an enveloped virus. The virion consists of an icosahedral core, likely formed from multiple copies of the core protein that encapsulates the viral RNA. The viral E1 and E2 glycoproteins are anchored in the core and the host cell derived double-layer lipid membrane which forms the outer envelope of the virion. In the serum, the virions have been observed either as free virions, bound to low-density lipoproteins (LDL) or very low-density lipoproteins (VLDL) or bound to antibodies (*66*). The bound LDL and VLDL particles and E2 play a role in the adhesion of the virion to the host cell by binding to heparan sulfate proteoglycans (HSPG), low-density lipoprotein receptor (LDLR) and scavenger receptor BI (SR-BI). Cellular entry is mediated by the tetraspanin CD81, Occludin and Claudin 1. CD81 and Occludin further determine the species tropism for HCV (*65*).

After viral entry the 9.6 kb genome is released into the cytoplasm, where it can directly serve as a template for viral protein translation. It contains one single open reading frame (ORF), flanked by 5' and 3' untranslated regions (UTRs). The ORF encodes one long polyprotein, that is afterwards further processed by cellular and viral proteases into 10 proteins: the three structural proteins E1, E2 and core and 7 non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. A further protein using an alternative reading frame has been described, but its function not well understood. It has recently been implicated in activation of DCs and T cells (*67*).

The 5'UTR of the viral RNA contains an internal ribosomal entry site (IRES), where the cellular ribosome can bind and translation being initiated, circumventing requirements for normal cellular protein translation initiation, such as the 5' cap (*65*).

The p7 protein is a transmembrane ion channel and is required for assembly and release of the free virions, but its exact mechanisms of action are unknown. The NS2 protein has cysteine protease functions and cleaves the polyprotein and the NS2-NS3 junction and is furthermore required for virion assembly.
The NS3 protein forms a complex with its cofactor NS4A. The NS3 protein has two domains, a protease and a helicase domain. The protease has been shown to be essential

for establishment of chronic infection by cleavage of important host cell factors for innate immune sensing, such as the RIG-I adaptor MAVS and TLR-3 adaptor TRIF. The helicase domain functions in viral replication.
NS4A is a multifunctional protein that serves to organise the replication complex and the formation of the membraneous web, the site of viral RNA replication (*65*).
NS5A is an indispensable part of the viral replication complex. Certain mutations in the NS5A protein have been linked to the virus' sensitivity to IFN, thus establishing a role of NS5A for the host antiviral response (*68*).
NS5B is the viral RNA-dependent RNA polymerase. It transcribes the ss(+)-strand RNA into a (-)-strand intermediate, which then serves as a template for the (+)-strand RNA genome (*65*).

3.3.2 Epidemiology and Therapy

World-wide an estimated 185 million people are chronically infected with HCV. That accounts for 2.5% of the world-population. Each year around 500 000 people die from HCV-related liver diseases. HCV is distributed globally with the highest prevalences in Central and East Asia and Africa (*69*). The seven genotypes have different global distributions. Genotype 1 is most common especially in developed countries, accounting for almost 50% of all HCV infections (*69*). In Europe genotype 3 and increasingly also genotype 4 are prevalent in drug-users, while genotype 2 is mostly seen in the mediterranean region (*70*).

Transmission takes place from human to human via blood, unlike other members of the flaviviridae, which are primarily spread via vectors (mosquitoes and ticks).
Until the establishment of HCV detection methods in the early 90s, blood transfusions were a major route of infection. Nowadays, high risk-behaviour includes intravenous drug usage and promiscuous sexual practices (*71*).

Acute infection is often asymptomatic and in many cases goes unnoticed. Those that develop acute symptoms can have fever, nausea, joint pain and jaundice. Only around 15-45% of acutely infected patients can clear the infection spontaneously, the other 55-85% will develop chronic infection. The probability for spontaneous clearance depends on a variety of factors such as age, sex, co-infection with other viruses, host genetic factors and viral genotype. Infection with HCV genoypte 1 is favorable for the outcome compared to non-type 1 infection (*72*). The virus is also more likely to be cleared the younger the patients are and when they are female (*73*).

Interest in NK cells in the resolution of HCV infection was sparked, when in 2004, Khakoo and colleagues found a genetic association between the expression of KIR2DL3 in patients homozygous for the HLA-C1 ligand and HCV clearance. They studied more than thousand patients who were exposed to HCV. 685 of those developed chronic infection and 352 cleared the virus. They found that those patients whose NK cells expressed the inhibitory KIR2DL3 homozygously on an HLA-C1/C1 background were more likely to clear the virus spontaneously. Of note, when stratified in terms of route of infections, this association only held true in patients with presumed low-dose viral inocculum, e.g. after a needle-stick, suggesting contribution of NK cells is only efficient when faced with low viral loads (*74*). A similar correlation was found in exposed but uninfected injection drug users. Their NK cells expressed higher levels of KIR2L3 than both drug users with chronic and resolved infections and healthy donors. The same study also found NKG2A less expressed on exposed, but uninfected drug users and could show that the ligand for NKG2A, HLA-E was upregulated in HCV-infected livers (*75*). However, there are also other studies which did not observe a correlation between KIR2DL3 and outcome of infection, albeit with smaller sample sizes and different patient cohorts (*76*) (*77*) (*78*).

At this time, the highest known impact for HCV outcome have number of single nucleotide polymorphisms (SNPs) in the IFN-λ (IL-28/29) gene locus. These IFN-λ variants have been implicated in the spontaneous clearance (*79*) and in IFN-α/Ribavirin treatment induced clearance (*80*), but the mechanism through which the SNPs affect infection outcome are not well characterized.

HCV therapy Until 2011 the standard approved therapy for HCV was pegylated IFN-α/ Ribavirin (peg-IFN/RBV). However, only 40-50% of patients in Europe and North America achieved sustained virological response (SVR) (no detectable HCV RNA 24 weeks after peg-IFN/RBV treatment). Of note, 99% of patients who achieve SVR remain completely virus free. While peg-IFN/RBV therapy had low success rates for genotype 1, other HCV genotypes especially genotype 2 achieved SVR rates of up to 80% (*70*). The low success rates combined with the severe side effects of the therapy demanded a high need for better treatment regimens. In 2011, the first direct acting antivirals (DAAs) were approved - telaprevir and boceprevir, both targeting the viral NS3/4A protease. These DAAs increased success rates, however, they still had to be administered together with peg-IFN/RBV (*70*). This changed in 2015 when several new DAA were approved for HCV, also in IFN-free therapies. DAA are grouped into four

classes depending on their target and their structure: The NS3/4 protease inhibitors, the NS5A inhibitors, the NS5B nucleoside polymerase inhibitors and the NS5B non-nucleoside polymerase inhibitors. Different combination treatments with several DAAs have achieved very high SVR rates in infections with all genotypes. In some cases almost 100% of patients achieved SVR (*81*).

3.3.3 Immunology

Innate immune response in hepatocytes

The primary sensing of HCV in hepatocytes is facilitated via RIG-I. Activation of RIG-I recruits the adaptor MAVS which activates IRF3 and IRF7 transcription factors, which amongst others induces the transcription of IFNs. Because of its important role in the activation of the innate immune response, the virus has established mechanisms to counteract the RIG-I pathway. The NS3-4A protease can cleave MAVS or release it from the membrane, where it cannot function.

Recognition via the TLR3- TRIF pathway has also been reported, but most likely only acts in combination with the RIG-I pathway. Some, but not all studies show that NS3-4A can also cleave TRIF.

Furthermore many other viral proteins are also implicated to interfere with host immune response, mainly with IFN signalling and its effects.

After sensing of the virus, cytokine, especially IFN production is induced which in turn induces ISGs. Interestingly, although ISG induction can be robustly seen in many systems (humans, chimpanzee, cell culture), in most no or only very little type I IFNs can be measured. However, type III IFNs (IFN-λs) were significantly upregulated. In fact, induction of ISGs might be primarily due to type III, and not type I IFN induction by viral sensing and its autocrine and paracrine signalling in hepatocytes. This could also be part of the explanation why SNPs in the type III IFN genetic region are so stongly predictive of spontaneous clearance and treatment response to IFN-α/ Ribavirin (*65*).

Adaptive immune responses

B cells HCV stimulates the production of HCV-specific antibodies from B cells. Although many antibodies are produced, the majority of those do not interfere with infection by neutralising the virus. Those that do mainly bind to specific regions of the E2 protein in the viral envelope. Most of these regions are important for binding to the entry receptors. Several studies have confirmed that antibodies can help to prevent infection of new host cells and support viral clearance. In one of them chimpanzees received anti-HCV antibodies before infection and some protection could be observed. Another study of patients from a unique cohort that was infected with HCV from one single source showed that patients with early antibody production had a better chance for viral clearance. Furthermore there seems to be selection pressure on the virus by B cells, as viral escape mutants for neutralising antibodies can be found in patients. Some of those mutants were even shown to use different entry receptors.

Although antibodies can help fight infections, it is also known that they are not necessary to do so, as agammaglobulinaemic patients who do not have mature B cells and therefore no antibodies, can also clear the virus (*65*).

T cells Both CD4 and CD8 T cells are important for viral clearance and in chimpanzees depletion of either leads to higher viral titres. The main effector cells are the CD8 T cells, nonetheless CD4 help is needed for CD8 T cell responses. Specifically in the acute stage of infection, a strong HCV-specific CD4 T cells response is observed in patients with viral clearance and certain MHC-II alleles are linked to better clearance. But in the chronic stage virus-specific CD4 T cells are hardly detectable any more.

CD8 T cells are the main effector cells against HCV. When they start to appear approximately 6-8 weeks after infection, there is a marked drop in the viral titre accompanied by a rise in liver enzymes. In particular their IFN-γ production is essential for the antiviral response. Perforin, on the other hand is less important and virus-specific CD8 T cells only contain little of it.

After the first drop in viral titre during acute infection, HCV quasispecies start to appear that escape from CD8 T cells, those species become enriched are likely to be important for the progression from acute to chronic phase. On top of the viral escape mutations from CD8 T cells, T cell dysfunction is a major contributor to persistence of infection.

CD8 T cells in chronically infected patients show an exhausted phenotype, low functionality and expression of inhibitory molecules, like PD-1, CTLA-4, CD160, KLRG1, Tim-3 and 2B4. Likewise, the few virus-specific CD4 T cells that are still present in chronic infection, show a phenotype similar to that of CD8 T cells. T cell exhaustion is however reversable, as successful DAA treatment can restore T cell function.

Another factor contributing to viral persistance is expansion of Tregs. In patient with chronic infection a higher Treg frequency is observed than in patients with resolved infection or healthy controls. Their main function is the suppression of CD8 T cells, primarily through cell-cell contact. Two mechanisms are described that induce the expansion of Tregs. First of all, infected hepatocytes produce TFG-β which supports the generation of Tregs. And second of all, there is evidence that Tregs also expand antigen-specifically, maybe even in response to the viral escape mutants. Consequently, the appearance of viral escape mutants does not only protect the virus from CD8 T cell responses, but at the same time induces Tregs and thereby further contributes to establishment of chronic infection. Interestingly, some viral proteins are more potent to induce Treg than others. For example immunisation with the Core protein induces Treg expansion, whereas immunisation with NS3 induces effector T cells.

Even though the expansion of Tregs supports the progression to chronic infection and limits anti-viral responses, in chronic infection they also protect from severe immunopathology, as patients with high Treg levels also have lower liver enzymes in serum (*65*).

3.3.4 Tools to study HCV

Cell culture systems

The Replicon System For years researchers tried to successfully propagate HCV in cell culture without luck. Until in 1999, Lohmann and Bartenschlager, utilized a strategy that has already been successful for other (+)-strand RNA viruses. They deleted genes which are not neccessary for replication (E1, E2, core, p7 and NS2) from the genotype 1b consensus sequence (Con1) and inserted a selection marker (neomycin resistance), creating a bicistronic HCV replicon. Electroporation of this *in vitro* transcribed viral RNA into Huh7 cells for the first time supported successful viral replication (*82*). Replication was then further enhanced by two processes. First, the virus acquired cell-culture adaptive mutations and second, highly permissive Huh7 subclones were selected.

This was done by electroporating the replicon, selecting positive cells with G418 and afterwards treating the cells with inhibitors or IFN to eliminate the virus. This way the Huh7.5, Huh7.5.1 and the Huh7-Lunet cells were established, which all replicate HCV with great efficiency.

The replicon-strategy and the cell-culture adaptive mutations were afterwards integrated into other HCV strains and genotypes, which also allowed their replication in Huh7 cells (*65*).

However, because all structural genes were missing and RNA has to be electroporated into the cells, the Replicon system could only be used to study certain aspects of the viral life cycle like replication, transcription or translation, but not attachment, entry, virion assembly and egress.

HCV pseudoparticles: HCV$_{pp}$ To study the HCV entry process, in 2003 two groups established retroviral pseudoparticles which display the E1 and E2 HCV envelope proteins on their surface. These viral particles are produced by 293T cells after transfection with plasmids encoding human immunodeficiency virus (HIV) or murine leukemia virus (MLV) gag-pol proteins and HCV E1 and E2 (*83*) (*84*). With the help of the HCV pseudoparticles (HCV$_{pp}$), many viral entry receptors could be identified.

Cell-culture derived HCV: HCV$_{cc}$ The same year, Kato et al. isolated a viral genotype 2a strain from a patient with fulminant hepatitis: japanese fulminant hepatitis 1 (JFH1) that was able to replicate at high titers in Huh7 cell without the previously known cell-culture adaptive mutations (*85*). Further investigations showed that this viral strain was also replicating when the full-length viral genome was used and that viral particles were formed. These were termed cell-culture derived HCV (HCV$_{cc}$) and could, for the first time, reproduce the complete viral life cycle.

Furthermore the JFH1 clone could not only replicate successfully in Huh7 cells and their derivatives, but also in many other human hepatic cell lines, and even in human non-hepatic and mouse cell lines (*65*).

One of the cell lines that was permissive for JFH1 (and also for Con1) was the Huh6 hepatoblastoma cell line, which was described by Windisch et al. in 2005 (*86*). The replication of JFH1-replicons was sensitive to treatment with IFN-α, similar to what

is observed in Huh7 cells. But interestingly, IFN-γ treatment, even at high concentrations, could not reduce viral levels in Huh6, whereas in Huh7 cells JFH1 RNA was greatly diminished. Further micro-array analysis revealed, that many ISGs were differentially regulated in Huh6 and Huh7 cell lines and at least one of them had functional consequences for the resistance to IFN-γ (*87*).

Identification of host cell factors In 2005 a novel role for miRNAs was discovered, when Jopling et al. described that miR-122 was necessary for HCV RNA replication by binding to the 5'UTR. The binding site is conserved in the different HCV genotypes, making miR-122 a promising drug target for HCV infections with all genotypes (*88*). In fact, a first clinical trial with miravirsen, a compound binding miR-122, was safe and showed promising results (*89*).

Another major break-through for the study of HCV in cell cultures was made in 2015, when Saeed et al. discovered that expression of the host cell factor SEC14L2 in Huh7.5 cells enables infection and replication of clinical HCV isolates. So far, only JFH1 was able to replicate without cell-culture adaptions and infect cells from virions (*90*).

HCV in primary hepatocytes Attempts to infect primary human hepatocytes (PHH) have been made, but with only moderate success. Replication in those cells is only very low, on top, these cells are very hard to come by and difficult to culture, as they loose their differentiation status quite fast in culture (*65*). A bit better are primary fetal human hepatocytes, as they are long-lived in cell-culture, but they also only support low-level replication and have only been used in very few studies (*91*).

Animal models

Chimpanzee model The chimpanzee (pan troglodytes) was the first animal model for HCV. It shares 98% genetic identity with humans and can be infected by non-adapted clinical isolates by intravenous injection of infected patient sera. The first infections of chimpanzees were already done a decade before HCV was even described, when it was still known as the causative agent of non-A, non-B hepatitis. The infection course is quite similar to humans. Many chimpanzees progress to chronic phase in which hepatitis occurs (as measured by elevated liver enzyme levels), but they rarely develop fibrosis, cirrhosis or hepatocellular carcinoma. Overall, HCV infection is less severe in chimpanzees than in humans.

The chimpanzee model has been an invaluable tool to study HCV, for testing HCV treatments (such as the DAAs or the miR-122) and for vaccine research. But due to ethical concerns, the use of chimpanzees is very restricted and even prohibited in many countries. It also comes with high costs, all of which limits availability. Although it remains the gold standard in HCV animal models, many of the studies can now be performed in the constantly improving mouse models.

Mouse models In recent years several mouse models have been established that address different needs in the study of HCV.

The first mouse models were transgenic mice that expressed different (only one, several or even the whole HCV polyprotein) viral proteins. These mice showed different liver pathologies and immune activation, giving insights into the different functions and immunogenicities of the viral proteins (*65*).

Infection of mice with free virus was made possible in genetically humanised mouse strains. Expression of human entry receptors in those mice overcame the species barrier and allowed for infection of the mice with free virus. In this model viral entry could be studied, as well as viral inhibitors and the mouse immune responses, as they show T and B cell responses (*92*). Recent genetically humanised mice additionally expressing host factors are now able to sustain chronic infection for more than a year and show liver inflammation and fibrosis (*93*).

Efforts have also been made to establish human-mouse chimeric models. The first models using immunodeficient mice to prevent graft rejection were the human liver chimeric mice, in which human liver cells were engrafted. These were useful to study the viral life cycle in human hepatocytes, but as they had no immune system, no immunological or vaccine studies could be made and mice showed no liver injury (*94*)(*95*). Subsequently a human immunocompetent liver mouse model was established in which mice were engrafted with human liver and hematopoetic stem cells. In this system, mice show liver inflammation and T cell responses to the virus (*96*).

3.4 NK cells in Hepatitis C Virus infection

NK are important players, especially in the intial phase of the anti-HCV immune response. The fact that NK cells do affect the outcome of HCV infection was again emphasised by the genetic association studies in 2004 already mentioned in chapter 3.3.2. It showed a strong association between the presence of KIR2DL3 on NK and the spontaneous resolution of infection in donors that were homozygous for the KIR2DL3 binding MHC-I (C1/C1) (*74*). The same year, it was also shown that IFN-γ produced by NK cells can in fact suppress replication of HCV RNA (*97*). Since then the mechanisms of how NK cells are activated during HCV infection and their phenotype and function during the different stages of infection have been studied, although often yielding quite controversial results.

3.4.1 Interaction of NK cells with HCV

Interaction of NK cells with HCV virions/ viral proteins Several early studies on the role of NK cells in HCV infection addressed the direct interaction of NK cells with HCV particles or with recombinant proteins.

In 1998 CD81 was identified as one of the binding receptors for HCV glycoprotein E2 (*98*) and subsequently it was shown that binding of E2 to CD81 on T cells serves as a co-stimulatory signal (*99*). CD81 is also expressed on NK cells, but two reports in 2002 showed that, in contrast to T cells, NK cells are inhibited by cross-linking of CD81 by plate-bound E2 or α-CD81 antibodies (*100*)(*101*). When HCV_{cc} became available Crotta et al. could confirm that also plate-bound HCV virions inhibited NK IFN-γ production via E2 - CD81 interaction. However, when free, non-plate-bound virions were used, even at concentrations that by far exceeded concentrations naturally occuring in infected livers or serum of patients, no effect on NK cell function was observed (*102*) (*103*). On the contrary, one group even showed that longer exposure of HCV_{pp} to isolated NK cells enhanced expression of activating receptors and NK cytokine production (*104*).

Nevertheless, these conditions are highly artificial and virion concentrations and NK interaction of this sort are very unlikely to occur *in vivo*.

Interaction of NK cells with infected hepatocytes Infection of a host cell induces many cellular stress responses that go along with upregulation of stress-related molecules on the cell surface and/or release of cytokines and chemokines that can be detected by NK cells.

One of the consequences of viral infection is altered MHC-I presentation. When Konan et al. expressed individual non-structural HCV proteins or protein precursors in 293T or Huh7 cells, they found that expression of the NS4A/B precursor protein alone, but also expression of full-length HCV RNA inhibited the ER-to-Golgi transport and thereby reduced antigen-presentation and MHC-I levels in Huh7 cells. (*105*). Likewise, Tardif et al. showed lower MHC-I levels on HCV replicon-carrying Huh7 cells because of aberrant folding due to ER stress caused by the virus (*106*). Herzer et al., on the other hand, showed that expression of Core protein alone, but also expression of the whole HCV polyprotein induced expression of MHC-I in HepG2, but not in Huh7 cells, which were used by Konan and Tardif et al., attributing it to the mutant p53 status of Huh7, but not HepG2 cells. They claim that the Core protein induces p53, which upregulates TAP1 and thereby increases MHC-I surface levels. When Core protein expressing HepG2 cells were used in a cytotoxicity assay against two NK cell lines, NK cytotoxicity was impaired compared to control cells (*107*). The two former studies did not address the effect of MHC-I regulation on NK cell function in their system. Of note, expression of the complete Core protein, but not E1, E2 or NS2 in HCV-protein transgenic mice led to enhanced hepatocyte killing that could be abogated by depletion of NK cells (*108*).

Although differences in MHC-I expression levels were observed in some studies, another study, using the HCV_{cc} infection system in Huh7.5 cells, showed no difference in MHC-I levels between uninfected and infected hepatocytes (*109*).

Beside the classical MHC-I molecules, the non-classical MHC-I molecules, especially HLA-E, are important for the development and function of NK cells. HLA-E is recognised by the inhibitory receptor NKG2A as well as by the activating receptor NKG2C, that is, however only expressed on a small subset of NK cells. HLA-E can be stabilised on the surface of cell lines incubated with certain peptides derived from HCV Core protein. This led to reduced cytolytic activity of a NK cell line. Notably, elevated HLA-E levels could also be shown on hepatocytes and Kupffer cells in patient livers (*110*).

Conflicting reports also exist about the induction of NKG2D ligands in HCV infection. One study claims downregulation of NKG2D ligands, another shows higher expression

of MIC proteins and third study observed no effects (*111*)(*112*)(*109*). Differences are likely due to the different experimental systems and viral strains used.

On the side of the NK cell it was observed that contact with HCV-infected hepatoma cells *in vitro* leads to the downregulation of activating receptors NKG2D and NKp30 which reduces the functional capacity of NK cells (*113*) (*114*).

Interaction of NK cells, accessory cells and infected hepatocytes Although NK cells can be affected possibly by the virus directly and by the infected hepatocytes, the immunological milieu and the interaction with other immune cells have a major influence on NK cell function. Especially monocytes/macrophages/Kupffer cells and DCs greatly influence NK cells after viral RNA sensing.

DCs can activate NK cells by expressing MIC-A/B after stimulation. This upregulation is inhibited in DCs from HCV-infected patients, resulting in less cytotoxic potential and IFN-γ production from NK cells after DC co-culture (*115*). Other NKG2D-Ligands, namely ULPB-1, -2 and -3 were not upregulated on myeloid DCs after HCV infection (*116*). Reversely, NK cells from HCV-infected patients also have a lower potential to activate DCs. This might be because NK cells from patients express high amounts of NKG2A that interacts with HLA-E on infected hepatocytes and induces NK production of IL-10 and TFG-β, which negatively regulates DC maturation and function (*117*).

The sensing of the virus by pDCs induces a strong type I IFN response and is dependent on TLR-7 and, surprisingly, on cell-to-cell contact. Free virus does not activate pDCs, but replicon-carrying hepatocytes that do not produce free viral particles can activate pDC IFN-α production (*118*). In a follow-up study the same group could show that the cell-to-cell contact dependent activation of pDCs is achieved via short-range transfer of viral RNA in exosomes (*119*). 24 h co-culture of peripheral blood mononuclear cells (PBMCs) with JFH1 infected Huh7.5 cells, performed by Zhang and colleagues, resulted in enhanced IFN-γ production by NK cells which could be almost completely abrogated by depletion of pDCs and was due to pDC IFN-α production (*120*). Exogenous IFN-α stimulation of NK cells has also been shown to upregulate TRAIL and TRAIL-dependent killing of replicon-carrying Huh7.5 cells (*121*).

Besides DCs, monocytes have also been implicated in the activation of NK cells in HCV infection. In fact, in the before mentioned study by Zhang et al., the authors could also show a role for monocytes in NK cell IFN-γ production. When they depleted pDC from PBMC - Huh7.5/JFH1 co-cultures, NK cell IFN-γ production was abrogated, but

isolated NK cells and pDCs co-cultured with infected hepatoma cells did not result in IFN-γ production. Only NK + pDCs + monocytes in co-culture with Huh7.5/JFH1 led to NK effector functions. The contribution of monocytes was attributed to their production and trans-presentation of IL-15, as monocytes from infected co-cultures show higher levels of IL-15Rα and blocking of IL-15 reduced NK IFN-γ production (*120*).

Production of IL-18 by monocytes in response to HCV infection was also implicated in NK activation by Serti et al. in 2014. In 24 h PBMC, but not NK co-cultures with Huh7/ JFH1 replicon cells, IFN-γ production was higher than in uninfected cultures. Depletion of monocytes and neutralisation of IL-18 abrogated the virus-dependent NK activation. The authors showed that IL-18 is induced in monocytes via detection of the HCV by the NALP3 inflammasome, as siRNA knock-down of NALP3 almost completely inhibits IL-18 production (*122*).

Accessory cells have not only been shown to aid NK cells effector functions, but can also inhibit NK cells and thereby contribute to NK dysfunction and establishment of chronic infection.

For example have Sène et al. shown that recombinant NS5A, as well as Huh7.5.1/JFH1 cell culture supernatants induce the production of the anti-inflammatory IL-10 and subsequently that of TGF-β by monocytes which downregulates NKG2D on NK cells and thereby limits their effector potential (*112*).

Goh et al. showed that after 1-week co-culture of PBMCs with HCV-infected Huh7.5.1 cells the myeloid compartment was enriched in myeloid-derived suppressor cells (MDSCs). Subsequent co-culture of the isolated CD33 $^{+}$ myeloid cells with autologous IL-12/18 stimulated NK cells for 2 days resulted in lower NK IFN-γ production. It is noteworthy that the percentage of IFN-γ producing NK cells was not significantly reduced and reduction in the per cell IFN-γ production (MFI) was only slightly, albeit significantly reduced (*123*).

3.4.2 NK cell responses during infection

The natural course of Hepatitis C Virus infection can be divided into three phases: first the incubation time where no viremia is present, followed by the acute phase in which the innate and the adaptive immune system is strongly activated and the virus tries to escape from its attack. If the immune system fails to control acute infection, around

6 month after infection the chronic phase is established that can last for decades if infection is not treated.

NK cells have been implicated as important players in all phases of HCV infection. However, many of the results are ambiguous. One problem of many of the HCV patient studies which makes them hard to compare to each other and to reproduce findings are the different cohorts that are studied and the often small sample sizes, especially in the acute phase of infection. Differences in viral genotype, patient ethnicity, which has a great influence on HCV outcome, age, sex, route of infection, but also differences in study design and choice of control groups likely all contribute to the observed discrepancies regarding NK cells in HCV infection.

NK cells in acute infection

To study NK cells during acute infection is not an easy task, because relevant patient cohorts are hard to identify, mainly because acute infection is clinically often silent and therefore remains unnoticed. Most studies were therefore conducted on high risk groups such as intravenous drug users (IDUs) and exposed healthcare workers, but those have generally very small sample sizes.

Several studies have followed IDUs who were exposed to HCV and either remained uninfected (exposed-uninfected) or progressed to acute infection. Those that progressed to acute infection, can furthermore be classified in two groups, one that subsequently resolved the virus (acute-resolving) and one that progressed to chronic disease (acute-chronic). Reports about the phenotype and function of NK cells in exposed-uninfected individuals vary greatly. For example, some reports say that NKp30 expression is higher on exposed-uninfected NK cells (*124*)(*125*), while another claims that there is no difference in NKp30 levels (*126*). Alter et al. observed that in patients that progressed to chonic disease NKp30 levels on NK cells were in fact higher than in patients with acute-resolving infection.(*127*)

Reports on NK function are equally conflicting. Degranulation of NK cells against standard targets (K562) or directly *ex vivo* was shown to be higher in exposed-uninfected individuals in one study (*124*), but lower in others (*126*) (*125*). Likewise IFN-γ production was reported to be higher (*125*), but also unchanged (*124*) (*126*) in exposed-uninfected compared to exposed-infected individuals. Although when compared to healthy controls IFN-γ production was lower in all exposed groups (*126*).

One study followed 12 health care workers who had been exposed to HCV by accidental needle sticks. 11 out of 12 remained uninfected and those showed a strong NK cell response early in infections. IFN-γ, degranulation, TRAIL, NKp44 and NKG2A expression were all induced shortly after HCV exposure when compared to follow-up, after virus clearance. Furthermore NK activation was seen around 4 weeks earlier than in the person that became infected and magnitude of NK degranulation correlated with the subsequent strength of T cell responses (*128*).

When comparing acute-resolving vs. acute-chronic infection instead of exposed-uninfected vs infected, it was observed that no matter the outcome, NK IFN-γ production, degranulation and NKG2D expression are higher in acute infected NK cells than in healthy individuals (*129*).

NK cells in chronic infection

Studies on NK cells in chronic infection are more numerous and with bigger sample sizes and some have also had access to intrahepatic, not only peripheral NK cells, but unfortunately this does not make their findings less conflicting than those from the acute phase of infection. In general it has to be taken into consideration that the immune system in the chronic phase of infection has already failed to clear the virus and that it is now acting in a state of chronic inflammation which is known to cause dysfunction or anergy of leukocytes. It is therefore hard to distinguish whether changes in phenotype or function are the cause or the consequence of chronic infection (*130*).

In terms of NK receptor expression during chronic infection, studies have reported both increase and decrease for many important NK receptors, such as NKG2D, NKp46 or NKp30 (*131*). However, in all that discrepancy, there is one receptor - NKG2A - which has consistently been reported to be upregulated in chronic HCV patients (*117*)(*132*)(*133*). Accordingly the ligand HLA-E also seems to be upregulated on infected hepatocytes as already mentioned in chapter 3.4.1 (*110*)(*132*). The relevance of this regulation have yet to be fully determined. Another NK marker that received more substantial attention was NKp46. Most studies have reported an increase in NKp46 on peripheral and intrahepatic NK cells in chronic HCV infection. This NKp46^{high} NK cell subset shows a strong cytotoxic and cytokine producing potential and can kill infected hepatocytes (*134*). This is substantiated by the finding that high number of intrahepatic NKp46^{+} cells correlate with higher liver inflammation scores (*135*).

Functionally most studies report that NK cells tend to increase cytotoxicity and decrease IFN-γ production during HCV. Mechanistical studies attribute this functional scewing to consistent exposure to IFN-α in the chronically inflammed liver which induced STAT-1 over STAT-4 phosphorylation and thereby favors NK cytotoxicity over IFN-γ production (*136*)(*137*). This could contribute to the persistance of virus, as IFN-γ is thought to be more important for the anti-viral response than cytotoxicity and at the same time contribute to live pathology. On the other hand, NK cells and especially the NKp46^{high} NK cells, have also been shown kill hepatic stellate cells and thereby contribute to reducing liver fibrosis. A further mechanisms to eliminate hepatic stellate cells is via NK expression of TRAIL (*134*)(*138*).

Of note, although conventional CD56^{+}CD3^{-} NK cells are pushed towards cytotoxicity, there is a CD56^{-} NK cells subset, defined as CD14^{-}, CD3^{-}, CD19^{-}, CD56^{-}, CD16^{bright}, emerging during chronic HCV, but also during many other pathogenic circumstances, that has shown to be highly dysfunctional (*139*).

Taken together, NK cell phenotype and functions are altered during chronic infection, a highly dysfunctional CD56^{-} NK subset appears and NK cells can contribute to liver pathology, but also to amelioration of fibrosis.

NK cells in treatment

For a long time IFN-α/Ribavirin treatment was, and in many parts of the world still is, the standard HCV therapy. But in the last few years the DAAs have revolutionised treatment, achieving almost 100% success rates for many viral genotypes.

As IFN-α is a potent activator of NK cells, many studies have addressed NK function during HCV therapy and studied the effect of IFN-α on NK cells in regard to their anti-HCV functions. Here, the majority of papers is in accordance that a strong and early NK cells response, as expressed for example in higher expression of activation marker CD69, predicts high chances of achieving SVR. IFN-α activates STAT1 phosphorylation and promotes NK cell cytotoxicity which coincides with rising alanine aminotransferase (ALT) levels as early as 24 h after treatment (*131*). It furthermore induces TRAIL expression on NK cells which is inversely correlated with HCV-RNA levels, suggesting TRAIL-mediated killing as an additional mechanism to the granzyme/perforin-mediated killing to eliminate virus-infected cells during therapy (*121*).

NK cells were also studied during IFN-free DAA therapy, although the DAAs do not act directly on the immune system this setting provides a chance to study the normalization of immune cells when the driver for a chronic infection, in this case the virus, is elminiated.

After successful DAA therapy, NK cell phenotype quickly normalises again, revealing that the dysfunction and distorted phenotype during the chronic infection did most likely not induce permanent changes in the NK cell subset (*140*).

Antigen-specific CD8 T cells also regain normal phenotype and function after successful DAA therapy but interestingly mucosal-associated invariant T cells (MAITs) seem to be irreversibly altered during HCV infection, the exact mechanism however remains to be determined (*141*).

4 Aim of this study

HCV infection is a major global health problem and incidences of HCV-related liver diseases are predicted to rise over the next years, despite new effective treatments.

Upon infection a minority of patients can spontaneously clear the virus, while most progress to chronic infection. Furthermore response to IFN-α/Ribavirin treatment is also only effective in some of the treated patients. The exact underlying mechanisms are still unclear, but several immune system-related factors have been implicated. Among those genetic associations with NK cell receptors.

However, studies on the phenotype of NK cells in HCV infected individuals are highly controversial and mechanistic insights into the interaction of virus-infected cells and NK cells are rare.

In the present study, we therefore wanted to investigate mechanisms of how HCV-replicating hepatocytes interact with NK cells directly and in the context of the immunological environment.

Results from this study are potentially relevant for therapeutic intervention strategies that increase NK cell functionality and anti-viral immune responses against HCV.

5 Material & Methods

5.1 Material

5.1.1 Chemicals

Table 5.1: Chemicals

Chemical	Company
Antibody diluent	Dako Cytomation
Bafilomycin	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich
Chloroform	Sigma-Aldrich
DTT	Sigma-Aldrich
EDTA	Ambion
Ethanol	Sigma-Aldrich
Fluoromount-G^{TM}	eBioscience
GW4869	Sigma-Aldrich
H_2O_2	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Mayer hemalum solution	Applichem
Methanol	Sigma-Aldrich
Nuclease-free water	Ambion
nucleoside triphosphates (NTPs)	ChemCyte
Phenol	Sigma-Aldrich
RNasin	Promega
Spermidin	Sigma-Aldrich
Tween20	Sigma-Aldrich
Xylol	Sigma-Aldrich

5.1.2 Enzymes

Table 5.2: Enzymes

Enzyme	Company
MluI restiction enzyme	New England Biolabs
RNase-free DNase	Promega
T7 RNA polymerase	Promega

5.1.3 Cell culture products

Table 5.3: Cell culture products

Product	Company
ATP	ChemCyte
Biocoll separating solution	Biochrom
Dimethyl sulphoxide DMSO Hybri-Max	Sigma-Aldrich
Dulbecco's modified Eagle's Medium	Sigma-Aldrich
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich
Fetal Calf Serum	gibco
Geneticin (G418)	gibco
Glutathion	Sigma-Aldrich
GolgiPlug	BD Biosciences
GolgiSTOP	BD Biosciences
HEPES buffer solution 1M	PAA
Hygromycin B	invitrogen
IL-12	Peprotech
IL-15	Peprotech
IL-2	NIH
LD Columns	Miltenyi Biotech
L-Glutamin 200mM (100x)	gibco
LS Columns	Miltenyi Biotech
MEM NEAA (100x)	gibco
Penicillin/Streptomycin	Sigma-Aldrich
Trypsin-EDTA Solution	Sigma-Aldrich

5.1.4 Kits

Table 5.4: Kits

Product	Company
CD14 microbeads human	Miltenyi Biotech
CD3 microbeads human	Miltenyi Biotech
Foxp3 / Transcription Factor Staining Buffer Set	eBioscience
human IL-18 ELISA Kit	R&D
Light-Cycler 480 SYBR Green I Master Kit	Roche
MACSplex human Cytokine 12 Kit	Miltenyi Biotech
NK cell isolation kit human	Miltenyi Biotech
NucleoSpin ® Gel and PCR Clean up Kit	Macherey Nagel
ProtoScript M-MuLV First Strand Synthesis Kit	NEB
RNeasy Mini Kit	Qiagen
TURBO DNase Kit	Ambion
VECTASTAIN Elite ABC Kit (Mouse IgG)	Vector Laboratories

5.1.5 Cell lines

Table 5.5: Cell lines

Cell line	Cell type	Medium
Huh6	hepatoblastoma	DMEM, 10 %FCS, 1% PS, NEA
Huh6 cure	hepatoblastoma	DMEM, 10% FCS, 1% PS, NEA
Huh6-sgJFH1	hepatoblastoma	DMEM, 10% FCS, 1% PS, NEA, 500 μg/ml G418
Huh7	hepatocellular carcinoma	DMEM, 10% FCS, 1% PS, NEA
Huh7 9-13	hepatocellular carcinoma	DMEM, 10% FCS, 1% PS, NEA, 1 mg/ml G418
Huh6-DENV	hepatoblastoma	DMEM, 10% FCS, 1% PS, NEA, 75 μg/ml hygromycin
K562	myelogenous leukemia	RPMI, 10% FCS, 1% PS

5.1.6 Antibodies

Table 5.6: Antibodies for flow-cytometry I

Antigen	Clone	Company
4-1BBL	282220	R&D Systems
Axl	108724	R&D Systems
B7-H6	1.18	G.Moldenhauer, DKFZ
CD107a	H4A3	BioLegend
CD112	TU27	BioLegend
CD127	A019D5	BioLegend
CD14	HCD14	BioLegend
CD155	SKII.4	BioLegend
CD158a/h	HP-MA4	BioLegend
CD158b	DX27	BioLegend
CD25	BC96	BioLegend
CD28	CD28.2	BioLegend
CD3	SK7	BioLegend
CD38	HIT2	BioLegend
CD40	5D3	BioLegend
CD56	HCD56	BioLegend
CD57	HCD57	BioLegend
CD80	2D10	BioLegend
CD86	IT2.2	BioLegend
CD94	HP-3D9	BD Bection Dickinson
CD96	NK92.39	BioLegend
DNAM-1	DX11	BioLegend
Dtk/ Tyro-3	96201	R&D Systems
Gal-9	9M1-3	BioLegend
GITR	621	BioLegend
HVEM	00122	BioLegend
IFN-γ	B27	BioLegend
MIC-A	AMO-1	Bamomab
MIC-B	M369	non-commercial

Table 5.7: Antibodies for flow-cytometry II

Antigen	Clone	Company
NKG2A	REA110	Miltenyi Biotec GmbH
NKG2C	134591	R&D Systems
NKG2D	1D11	BioLegend
NKp30	P30-15	BioLegend
NKp30-Fc	-	R&D Systems
NKp44	P44-8	BioLegend
NKp44-Fc	-	R&D Systems
NKp80	5D12	BioLegend
OX40	Ber-ACT35	BioLegend
OX40L	11C3.1	BioLegend
pan-MHC class I	w6/32	BioLegend
PD-L1	MIH1	BD Becton Dickinson
TIGIT	MBSA43	eBioscience
TIM-3	344823	R&D Systems
ULBP-1	M291	non-commercial
ULBP-2	M311	non-commercial
ULBP-3	M550	non-commercial
ULBP-4	M479	non-commercial

Table 5.8: Secondary antibodies for flow-cytometry

Secondary antibody	Company
goat α-human-Fc	Jackson ImmunoResearch Laboratories
goat α-mouse-Fc	Dianova

Table 5.9: Isotype control antibodies for flow cytometry

Antigen	Clone	Company
CD99-Fc	-	R&D Systems
mIgG1	MOPC-21	BioLegend
mIgG2a	MOPC-173	BioLegend
mIgG2b	MPC11	BioLegend
rIgG2b	10H5	eBioscience

Table 5.10: Neutralising Antibodies / Reagents

Antigen	Clone	Company
GM-GSF (LEAF)	BVD2-23B6	BioLegend
IL-18 BPa Fc	-	R&D Systems
IL-2 (LEAF)	MQ1-17H12	BioLegend
IL-6 (LEAF)	MQ2-13A5	BioLegend
OX40L	MM0505-8S23	antikoerper-online
OX40L	Oxelumab R4930	absolute antibody

Table 5.11: Antibodies for Immunohistochemistry

Antigen	Clone	Company
msOX40L	MM0505-8S23	abcam
msIgG1	MOPC-21	BioLegend
horse αms-biotin	-	Vector Laboratories

5.1.7 Cell dyes

Table 5.12: Cell dyes

Bye	Company
7-AAD	BioLegend
CFSE	Sigma-Aldrich
Zombie Aqua^{TM}	BioLegend

5.1.8 Oligonucleotide RT-qPCR Primers

Primer	Sequence 5' - 3'
HCV RNA gt2a forward	TCTGCGGAACCGGTGAGTA
HCV RNA gt2a reverse	GGGCATAGAGTGGGTTTATCCA
ULBP-1 forward	AAGAATTTTTGATGTACTGGGAACAA
ULBP-1 reverse	ATGAAGCAGAGGAAGATGATGAGAA
ULBP-2 forward	GATGGCTTGAGGACTTCTTGATG
ULBP-2 reverse	TGGGTTGTGCCTGAGGACAT
MIC-B forward	TGCAGAAACTACAGCGATATCTGAA
MIC-B reverse	TGCATGTCACGGTGATGTTG
B7-H6 forward	TTATTCCCAACCCCTCAACA
B7-H6 reverse	AGACACAATGGCTCCAGGTC
CD155 forward	TCAACGCCAAGTCATCCTCC
CD155 reverse	CCAAGAGTTCAAGACCAGCC
NKp44L forward	ATCTGGTTTCGGACGGACTG
NKp44L reverse	ATTGCTTCCCACAAAGTTGCC
CD54 forward	TGGTAGCAGCCGCAGTCATA
CD54 reverse	CTCCTTCCTCTTGGCTTAGT
GAPDH forward	GCAAATTCCATGGCACCGT
GAPDH reverse	TCGCCCCACTTGATTTTGG

5.2 Methods

5.2.1 Generation of Replicon cell lines

***In vitro* RNA transcription of subgenomic RNAs** In vitro transcripts were generated by using 10 μg DNA from the plasmids in figure 5.1 A)-C) that had been linearized by 1 h digestion with the restriction enzyme MluI. DNA was purified using NucleoSpin ® Gels and PCR Clean up Kit. In vitro transcription reaction mixtures (final volume 100 μl) contained 80 mM HEPES (pH 7.5), 12 mM MgCl2, 2 mM spermidine, 40 mM dithiothreitol (DTT), 3.125 mM of each nucleoside triphosphate, 1 U of RNasin/ μl, 0.1 μg of plasmid DNA/ μl, and 0.6 U of T7 RNA polymerase/ μl. After 2 h incubation at 37°C, 0.3 U of T7 RNA polymerase/μl of reaction mixture was added and the reaction mixture was incubated over night at 37°C. 1.2 U of RNase-free DNase per μg of plasmid DNA was added to terminate transcription and incubated for 30 min at 37°C. RNA was extracted with acidic phenol and chloroform, precipitated with isopropanol at room temperature and dissolved in RNase-free water.

Electroporation of subgenomic RNAs Huh6 or Huh7 cells were trypsinised and washed with PBS and resuspended at single-cell suspensions at a concentration of 1.5x10^7 cells/ml in Cytomix (described in (*142*)) supplemented with 2 mM ATP and 5 mM glutathione for subsequent electroporation. 2.5 μg in vitro RNA transcripts were mixed with 100 μl cell suspension and transfected by electroporation using the Bio-Rad GenePulser system with 0.2 cm gap cuvette at 500 μF and 166 V. Cells were immediately diluted in DMEM + 10% FCS + 1x L-Glutamin + 1% Penicillin/Streptomycin and seeded.

Selection and curing of transfected cells 2 days post electroporation 500 μg/ml (Huh6-sgJFH1) or 1 mg/ml (Huh7 9-13) G418 (Geneticin) or 75 μg/ml Hygromycin (Huh6-sgDENV) was added to the medium to select cells carrying the replicon. After the selection process was completed, some of the cells were treated with viral inhibitors (100 nm BMS-553 and 5 μM Telaprevir) for 2 weeks to cure cells from viral RNA. Elimination of viral RNA was confirmed by RT-qPCR. Culture medium of replicon cell lines was always supplemented with the above mentioned antibiotics.

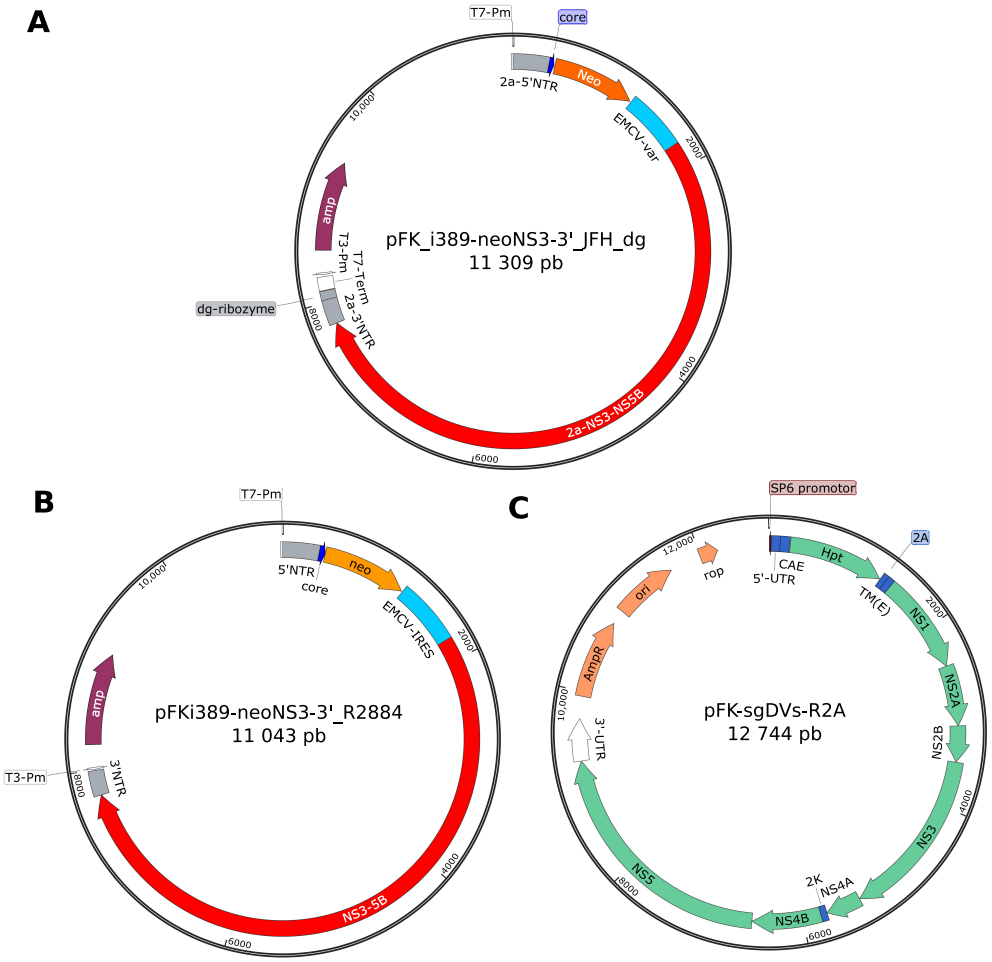

Fig. 5.1: Plasmids containing the HCV and DENV replicons Plasmid of the replicons were kindly provided by Ralf Bartenschlager. **A)**Plasmid map of the sgJFH-1 replicon. **B)** Plasmid map of the sg9-13 replicon. **C)** Plasmid map of the sgDENV replicon.

5.2.2 Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA isolation $1x10^6$ cells were harvested and washed with PBS for RNA lysis using the RNease Mini Kit. The cell pellet was resuspended in 350 μl RLT buffer supplemented with 3,5 μl β-mercaptoethanol. Samples were homogenized by adding them to QIA shredder columns and centrifugation for 2 min at full speed. To the flow-through 350 μl 70% ethanol was added and the whole lysate added onto RNeasy spin columns, which were centrifuged at full speed for 15 s. Columns were washed three times: 1x with 700 μl of buffer RW1 and 2x with 500 μl buffer RPE, each time centrifugation was

15 s at full speed. Membrane was dried by centrifuation for 2 min at full speed. RNA was eluted from the column by adding 50 μl nuclease-free water and centrifugated for 2 min.

DNase digestion DNase was digested using the TURBO DNase Kit. 10 μg of isolated RNA + 5 μl 10xTURBO buffer + 1 μl DNase + necessary amount of nuclease-free water to reach 50 μl were incubated at 37°C for 30 min. DNase was inactivated by adding 5 μl of DNase inactivation reagent and incubation at room temperature for 2 min. RNA sample was centrifuged and supernatant transferred to a fresh tube.

First strand cDNA synthesis 5 μl of the DNase treated-RNA were incubated with 3 μl of random primer provided in the ProtoScript M-MuLV First Strand Synthesis Kit at 70°C for 5 min to denature RNA. To the reaction 10 μl M-MuLV Reaction Mix and 2 μl nuclease-free water was added and first strand cDNA synthesis carried out at 42°C for 1 h. Enzyme was activated by incubation of the reaction at 80°C for 5 min.

RT-qPCR The 20 μl cDNA reaction was diluted by adding 80 μl of nuclease-free water. 2.5 μl of diluted cDNA were added per well to LightCycler480 Mulitwell plates together with 1.5 μl nuclease-free water, 0.5 μl forward and 0.5 μl reverse primers in a final concentration of 0.5 μM and 5 μl of Master Mix from the LightCycler 480 SYBR Green I Master Kit. Each reaction was performed in triplicates. As negative control nuclease-free water was used instead of cDNA. The plate was sealted with foil, shortly centrifuged and the reaction run with the following PCR program on the Roche LightCycler 480:

Preincubation		95°C	7 min
Amplification	Denaturation	95°C	10 s
	Annealing	60°C	15 s
	Extension	72°C	20 s
	45 cycles		
Melting curves		95°C	5 s
		65°C	60 s
Cooling		40°C	10 s

5.2.3 Flow cytometry staining and analysis

Extracellular staining For extracellular staining of cells for flow cytometric analysis, cells were harvested with Trypsin and washed with PBS. Between 1x10^5 and 1x10^6 cells were transferred into 96-well U-bottom plates and resuspendend in 25 μl FACS buffer (PBS + 3% FCS + 0.05% NaN_3) containing all primary antibodies, isotypes or fusion proteins. Cells were incubated for 30 min on ice in the dark and then washed twice with FACS buffer. In case of unconjugated primary antibodies, fluorophor-conjugated 2nd antibodies were added in 25 μl FACS buffer for 30 min on ice in the dark, followed by two washing steps using FACS buffer. For staining of dead cells, 7-AAD dye was added to the cells for 5 min diluted 1:50 in FACS buffer followed by two washing steps using FACS buffer before analysis. If cells were fixed for intracellular staining, AquaZombie was used for live/dead cell discrimination instead of 7-AAD. For this, AquaZombie dye was diluted 1:250 in PBS and 25 μl added to the cells for 10 min on ice and then 25 μl of primary antibodies diluted in PBS (concentration adjusted for a final volume of 50 μl) and directly added to the cells for 30 min without washing.

Intracellular staining For intracellular staining cells were fixed and permeabilised after AquaZombie and extracellular staining using the Foxp3 / Transcription Factor Staining Buffer Set from eBioscience. Fixation/Permeabilization concentrate was diluted 1:4, 25 μl added to the cells and incubated for 30 min on ice. Primary antibodies were diluted in Permeabilization buffer at the titrated concentrations and 25 μl added to cells for 30 min on ice. Afterwards cells were washed twice with Permeabilization buffer and once with FACS buffer before analysis.

Cells were analysed on a BD FACS Calibur^{TM} or a BD FACS Canto II^{TM}. For compensation, single stainings of each used fluorophor were recorded. Data was analysed using the FlowJo9.9.4 (Treestar) software.

CFSE labelling of PBMCs PBMCs were adjusted to 1x10^7 cells/ml in PBS and 3.3 μl of 300 μM CFSE/ml added while shaking the cells. Cells were incubated for 10 min at 37°C in the incubator and then washed twice with complete DMEM medium.

5.2.4 Isolation of PBMCs and co-culture set-up

Isolation of PBMCs from human peripheral blood Buffy coats were ordered from the Blutbank Mannheim and diluted 1:4 with pre-warmed PBS. 15 ml of Bicoll separating solution was added to the bottom of a 50 ml falcon and carefully overlaid with 35 ml of the diluted buffy coat. Tubes were centrifuged at lowest acceleration and without brakes for 30 min at 1800 rpm. The cell layer was removed and cells washed 4 times with PBS.

Co-culture set-up The isolated cells were in some cases stained with CFSE, treated with neutralising agents and/or inhibitor GW4869 before co-culture. For neutralization cells were spun down and the pellet incubated for 20 min with the appropriate amount of antibody or isotype control (αGM-CSF: 7.5 μg/ml, αIL-6: 5 μg/ml, both αOX40L: 10 μg/ml, IL-18 BPa: 2.5 μg/ml, αIL-2: 20 μg/ml). Afterwards appropriate amount of medium to reach the final antibody and cell concentration (2x10^6 cells/ml) was added. Exosome-inhibitor GW4869 or equivalent amounts of the solvent DMSO was added to a final concentration of 10 μM. 2x10^6 PBMCs, NK cells or PBMCs depleted of $CD3^+$ or $CD14^+$ cells in 1 ml complete DMEM were added to 24-well plates containing the hepatocytes which were seeded one day before at concentrations to reach 70-80% confluence at the start of co-culture. Cells were then incubated for up to 6 days without medium exchange at 37°C, 5% CO_2. Neutralising reagents were again added at day 4.

For Transwell experiments, 4x10^5 isolated $CD14^+$ cells in 1 ml complete DMEM medium were added to the bottom well and 4x10^5 PBMCs depleted of $CD14^+$ cells added in 200 μl into the Transwell insert.

5.2.5 Magnetic-activated cell sorting (MACS)

Isolation of NK cells and $CD14^+$ cells and depletion of $CD3^+$ and $CD14^+$ cells was done using cell isolation kits (Miltenyi Biotech) according to manufacturer's protocol, although only 70% of the indicated antibody solutions was used. Purity of isolated cells and depletion efficacy, as determined by flow cytometry staining, was in all cases >95%, except for depletion of $CD3^+$ cells, where efficacy was only 80-85%.

5.2.6 Functional assays

Cell restimulation for intracellular IFN-γ staining

After the co-cultures PBMCs were harvested and washed with complete DMEM medium. Cells were resuspended in fresh medium containing 5 μg/ml IL-12 and 50 μg/ml IL-15. PBMCs harvested from one well of the 24-well plate were distributed to 3 wells of a 96-well plate (200 μl each) and incubated at 37°C for 18 h. For the last 4 h 50 μl of 1:166.66 in medium diluted GolgiPlug (containing Brefeldin A) was added to the wells. Afterwards cells were washed and stained for flow cytometric analysis.

CD107a degranulation assay

After the different co-cultures (Huh6, Huh6 cure, Huh6-sgJFH1 and PBMC only) PBMCs were harvested, washed with complete DMEM medium and adjusted to 1x10^6 cells/ml. Target cells (Huh6, Huh6 cure, Huh6-sgJFH1 and K562) were also harvested by trypsinisation and also adjusted to 1x10^6 cells/ml. 100 μl of PBMCs (effector cells) and target cells were added to a 96-well V-bottom plate (E:T ratio = 1:1). As a control effector cells were cultured alone in 200 μl final volume. 1 μl/well CD107a-FITC antibody was added to each well at the beginning of the assay and after 1 h 0.5 μl/well GolgiSTOP (containing Monensin) was added. After an additional 4 h cells were harvested and stained for CD3, CD56 and 7-AAD.

5.2.7 Cytokine measurement

MACSplex For determination of cytokines in co-culture supernatants, those were harvested at day 6 centrifuged and filtered to remove cells and cellular debris. Cytokine levels were analysed using the MACSplex Cytokine 12 Kit (Miltenyi Biotech) according to manufacturer's instructions.

IL-18 ELISA IL-18 was measured by ELISA (R&D) from centrifuged and filtered co-culture supernatants, harvested at day 6 according to manufacturer's instructions.

5.2.8 Immunohistochemistry staining

Paraffin embedded liver sections of HCV, HBV and healthy subjects were provided by the DZIF Biobank in cooperation with Dr. Felix Lasitschka (Project-No: i-0011). Sections were deparaffinised by placing them subsequently into baths with xylol (2x), 100% ethanol (2x), 96% ethanol, 70% ethanol each for 5 min and then into VE-water. We used heat-mediated antigen retrieval. This was done by placing the slides into boiling 1 mM EDTA solution at pH 8 for 20 min in a steam cooker. Afterwards slides and buffer was cooled for 20-30 min on ice. Slides were washed twice with VE-water and then placed for 20 min at room temperature into methanol supplemented with 2% of 30% H_2O_2 to block endogenous peroxidase activity. Methanol was thoroughly washed away and liver sections on the slides circled with a grease pen. Sections were blocked with 10% Horse serum (Vectastain) + 0.1 mg/ml hIgG (Sigma) in antibody diluent (DAKO) for 20-30 min at room temperature. Afterwards, the primary antibody or isotype control was diluted (OX40L 1:25) in antibody diluent and added to the slides overnight on 4°C. The next day slides were washed 3x 5 min in PBS + 0.1% Tween20 and then incubated with the secondary biotinylated antibody (Vectastain Elite ABC Kit) also diluted in antibody diluent for 30min at room temperature. Antibody was washed away 3x 5min in PBS + 0.1% Tween20. The AB-Komplex (1 part A - 1 part B solution) from the Vectastain Elite ABC Kit which had already been incubated for 30 min alone was then added to the slides for 30 min on room temperature and then washed away 3x 5 min in PBS + 0.1% Tween20 and 1x3 min in PBS. The DAB-Chromogen was added to the slides until the desired staining intensity was reached before the reaction was stopped by putting the slides in VE-water. To counter stain the tissue, the slides were placed for a few seconds into mayer hemalum solution and then extensively washed with tap water and shortly in VE-water before mounting of cover slid with Fluoromount medium.

5.2.9 Statistical analysis

Statistical significance was calculated using paired two-tailed student's t test in the GraphPad Prism software: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$. All combined bar graph data depict mean + SEM.

6 Results

The HCV replicon system has been a valuable tool to study various virological, cellular and immunological aspects of HCV infection *in vitro* since its development in 1999 by Lohmann and Bartenschlager (*82*). By far the most commonly used cell line for the replicon system are the Huh7 and its subclone Huh7.5 hepatocellular carcinoma cell lines. However, studies in the past have shown that upon infection, none of the tested NK cell ligands were regulated and that NK cells could recognise and kill infected and uninfected Huh7.5 cells to the same degree (*109*). Therefore, we established a new system using the replicon of HCV genotype 2a: JFH1(japanese fulminant hepatitis 1) and the Huh6 hepatoblastoma cell line, which has been shown to support HCV replication before (*86*).

6.1 The Huh6 HCV Replicon System

6.1.1 Establishment of the Huh6 Replicon System

Figure 6.1 A shows the set-up of the Huh6 HCV replicon system and its subsequent co-culture with PBMCs to study the interaction of replicon-carrying hepatocytes and immune cells.

We used HCV replicons deleted of all structural genes, therefore unable to produce free virions and instead equipped with a neomycin resistance gene. This subgenomic replicon (sg-JFH1) was electroporated into the hepatoblastoma cell line Huh6 and positive clones selected with 500 μg G418 (Geneticin). Afterwards we treated some of the positive Huh6-sgJFH1 cells with viral inhibitors BMS-553 (Daclatasvir-like inhibitor of NS5A) and Telaprevir (inhibitor for viral protease NS3-4A) to prevent viral replication and cure the Huh6 cell from the sg-JFH1 replicon, thereby establishing the 'Huh6 cure' cell line. The Huh6 cure cell line controls for possible changes due to electroporation and G418 selection.

HCV RNA could only be detected in the Huh6-sgJFH1 cell line, but was absent in the parental Huh6 and the Huh6 cure line, while GAPDH levels were equal in all three lines (Fig. 6.1 B)

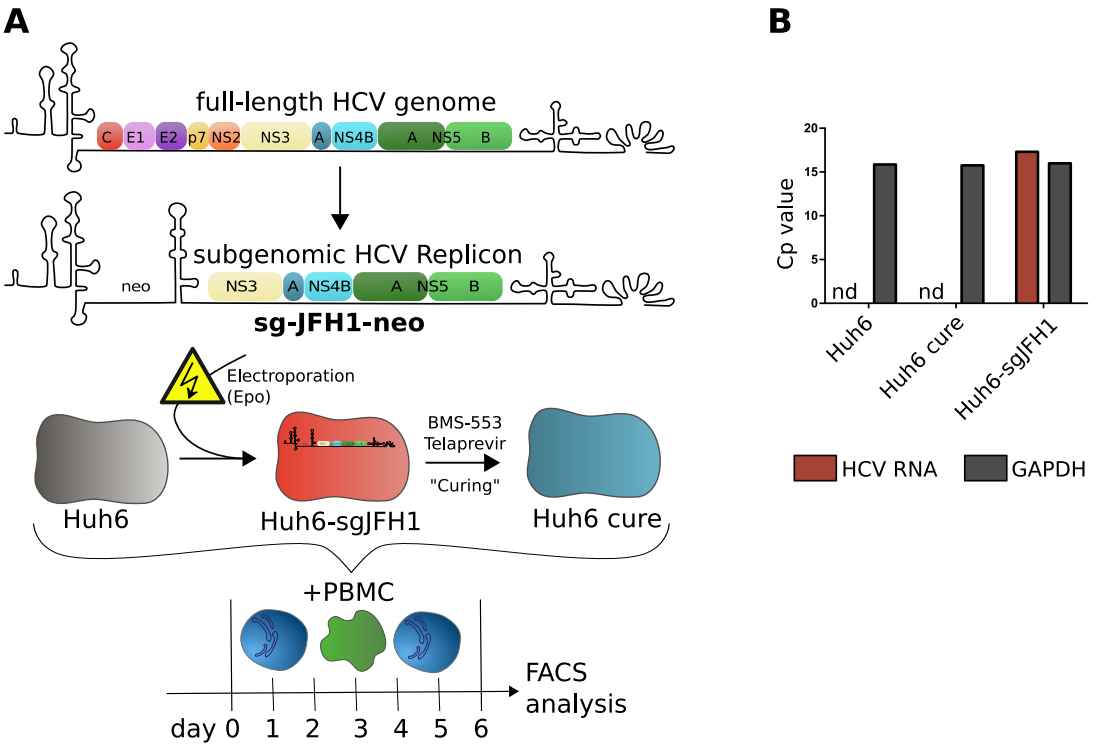

Fig. 6.1: The Huh6 Replicon system. Establishment of the subgenomic JFH1-neo HCV replicon system in Huh6 cells. **A)** Schematic of the replicon system and PBMC co-culture. Full-length HCV RNA from genotype 2a isolate JFH1 was deleted of core, E1/2, p7 and NS2 genes and supplemented with a neomycin-resistance gene and an additional IRES to create the subgenomic (sg)-JFH1-neo replicon. This replicon was electroporated into the Huh6 hepatoblastoma cell line to create the Huh6-sgJFH1 line and replicon positive clones selected with 500 μg/ml G418. Subsequent treatment of Huh6-sgJFH1 line with the direct antivirals BMS-553 (100 nM) and Telaprevir (5 μM) for 2 weeks created the Huh6 cure line. These 3 cell lines were used in co-culture experiments with PBMCs for up to 6 days, followed by flow cytometric analysis of PBMCs. **B)** qPCR analysis of Huh6, Huh6 cure and Huh6-sgJFH1 cells using HCV RNA-specific primers and GAPDH as control. nd=not detected, n=1

6.1.2 Characterisation of the Huh6 Replicon System

After the Huh6 replicon lines were established we characterized them regarding NK relevant surface proteins. Flow cytometry staining for 13 ligands of the most important known inhibitory and activating NK cell receptors, such as NKG2D, DNAM, NCRs and KIRs showed no significant alteration in any of the investigated proteins (Fig. 6.2 A). This observation was substantiated by results from RT-qPCR experiments, measuring

mRNA levels of 7 NK cell ligands. As shown in figure 6.2 B, none of the indicated NK cell ligands showed expression values that were significantly different between both Huh6 - Huh6-sgJFH1 and Huh6 cure - Huh6-sgJFH1.

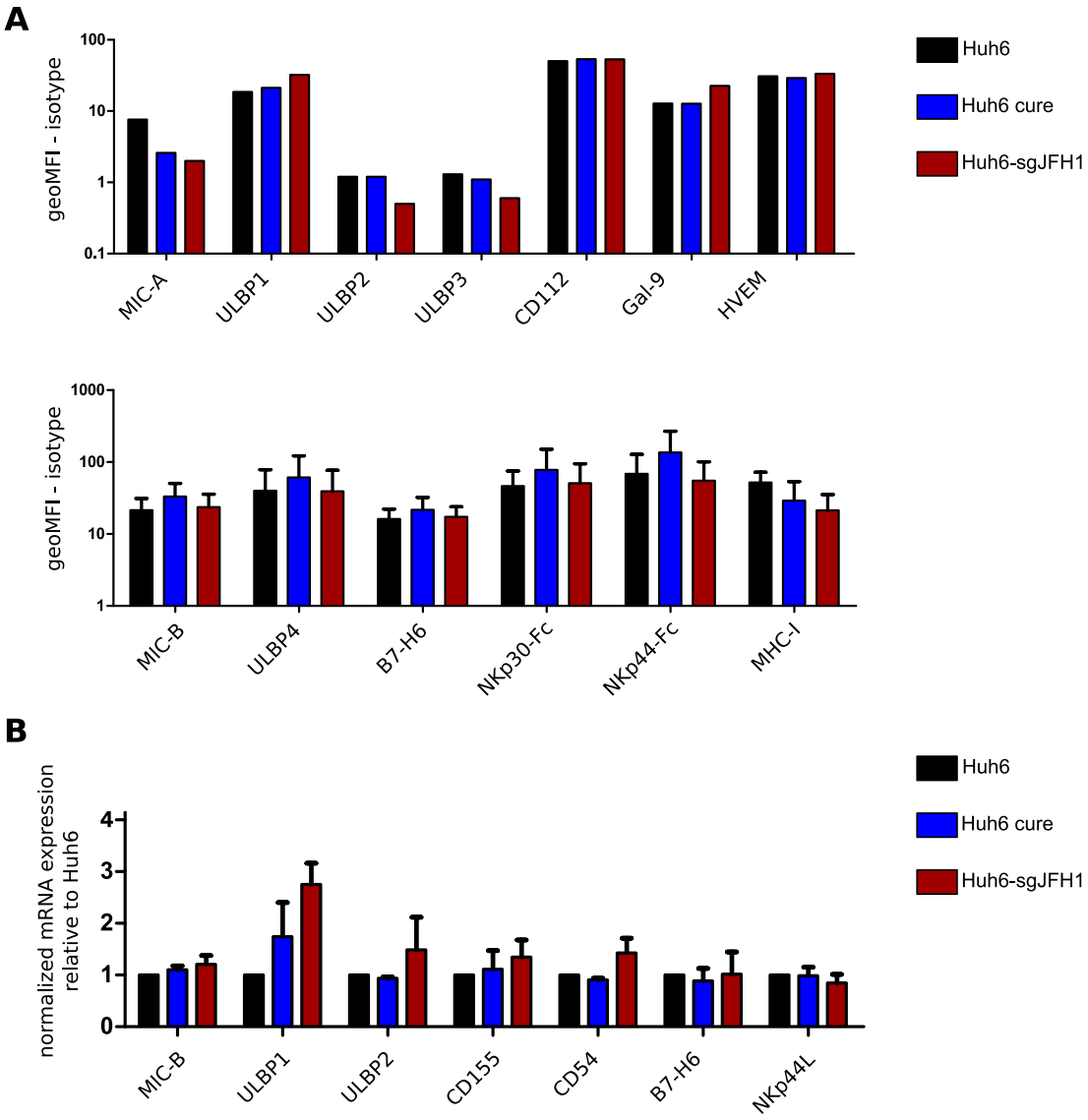

Fig. 6.2: Ligands for NK cells are not differentially expressed on Huh6, Huh6 cure and Huh6-sgJFH1 cell lines. A) Flow cytometric analysis of NK cell ligands on the indicated cell lines. Displayed are the isotype corrected geometric MFI values. Upper graph n=1, lower graph n=3-5. **B)** qPCR analysis of mRNA of indicated NK cell ligands on Huh6, Huh6 cure and Huh6-sgJFH1 cells lines. Displayed are values normalised to GAPDH expression, relative to expression in Huh6. n=2

6.2 NK phenotype in PBMCs - Huh6-sgJFH1 co-cultures

6.2.1 Screening NK cell surface markers after co-cultures

We set up co-cultures (4-6 days) between the parental, cured or sg-JFH1 Huh6 cells and PBMCs as described in figure 6.1 A to investigate possible effects of the replicon on NK cells. Even though none of the so far tested NK cell ligands were altered in the Huh6 cell lines by the HCV replicon, NK cells could be affected via various other mechanisms including so far unknown or untested ligands on the hepatocyte surface, cytokines secreted by the hepatocytes or other mononuclear leukocytes within the PBMCs after recognition of the viral RNA or cross-talk between NK cells and other immune cells. For the co-culture we first seeded the hepatocytes and the next day added freshly isolated PBMCs from buffy coats of healthy donors. After 4-6 days we removed the PBMCs from the wells and directly analysed them by flow cytometry or restimulated them for functional studies.

PBMCs were first gated according to forward/sideward scatter on the lymphocyte population, followed by exclusion of cell doublets and dead cells (i.e. 7-AAD negative or for fixed cells AquaZombie negative cells) and finally gated on $CD3^{-}CD56^{+}$ NK cells (Fig. 6.3 A).

Figure 6.3 B shows the results for one of two initial screening experiments wherein we stained an array of 6 inhibitory, 11 activating and 2 cytokine receptors on NK cells after the co-culture with either parental Huh6 of Huh-sgJFH1 cells. We found that the percentage of NK cells expressing these receptors was similar in both conditions for the majority of them. The same was true for the receptor expression per cell, the MFI, which was also unchanged in most cases (data not shown). However, there were 2 receptors that showed an increased percentage of expression in both experiments after co-culture with the Huh6-sgJFH1 line: the TNF receptor superfamily member OX40 and the high-affinity α-chain of the trimeric IL-2 receptor, CD25.

6.2.2 Phenotypical and functional changes after co-culture

After the initial screening of NK cells from Huh6 co-cultures with two donors we could confirm a highly significant upregulation of CD25 in 29 donors. A representative histogram and a summary of all donors, showing the increase of CD25 expression after Huh6-sgJFH1 co-culture compared to Huh6, Huh6 cure or PBMC cultured alone is

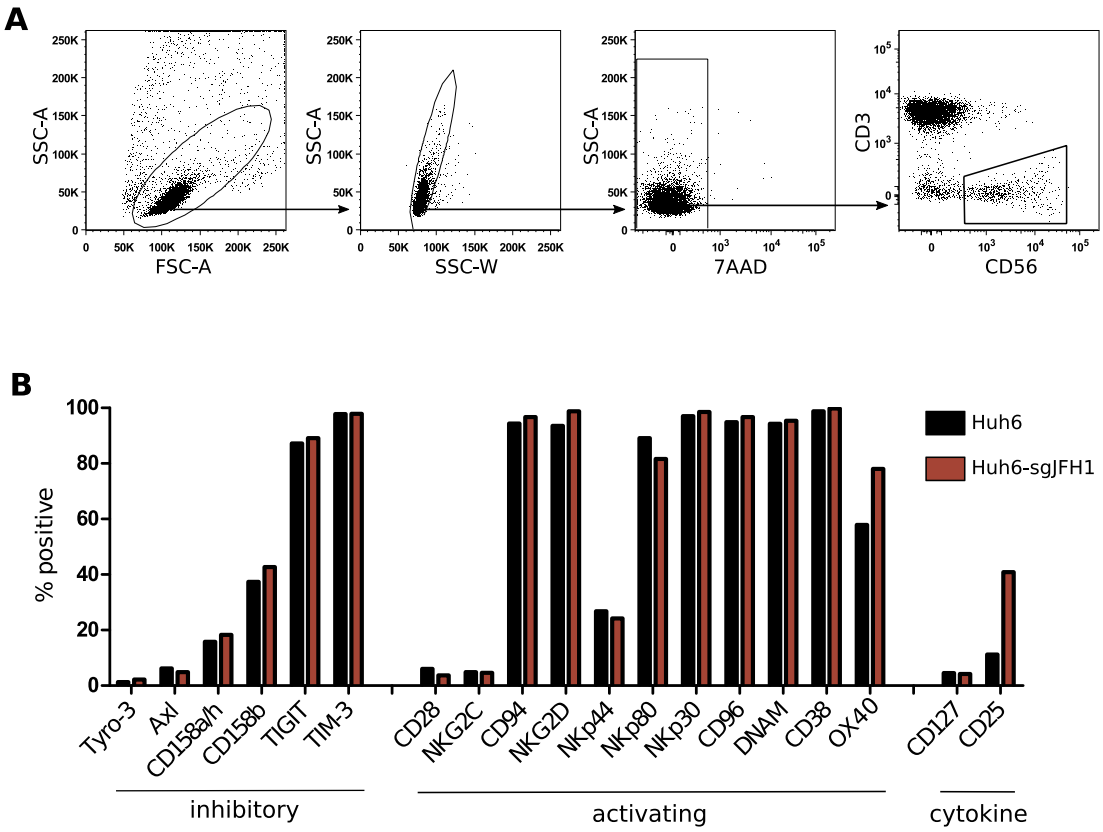

Fig. 6.3: CD25 and OX40 are upregulated on NK cells after co-culture with Replicon cells Freshly isolated PBMCs from healthy donors were co-cultured with Huh6 or Huh6-sgJFH1 cells for 4 days in medium supplemented with 100 U/ml IL-2. At the end of the co-culture, PBMCs were harvested and analysed by flow cytometry. **A)** Gating scheme of PBMCs after co-culture. NK cells were distinguished by gating on single, live (7-AAD$^-$), CD3$^-$ CD56$^+$ lymphocytes. **B)** Flow cytometry analysis of NK cells after 4 day PBMC - Huh6/ Huh6-sgJFH1 co-culture. Percentage of NK cells positive for the indicated receptor are shown. Data is representative for 2 donors.

shown in figure 6.4 A. The summary of all tested donors reveals that on average the percentage of CD25$^+$ NK cells is 22% +/- 2,3% or 26,5% +/- 2,5% after Huh6 or Huh6 cure culture, respectively, and that this is significantly increased to 39% +/- 3% after Huh6-sgJFH1 co-culture.

Because expression of CD25 endows the IL-2 receptor with high affinity binding to its ligand IL-2 which is an important pro-proliferative cytokine, we further investigated the effects of virus-induced CD25 upregulation on NK cells proliferation. This was done by labelling PBMCs before co-culture with the proliferation tracking dye carboxyfluorescein succinimidyl ester (CFSE). By every cell division, the amount of CFSE is divided into half, making it possible to track the number of cell division a cell has undergone.

Staining with CFSE before co-culture revealed that after 6 days significantly more NK cells in the replicon-containing co-cultures (38% +/- 3%) than in the parental (21% +/- 2%) or cured (21% +/- 3%) conditions had proliferated.

Finally cytokine production and cytotoxic potential of NK cells was investigated. After co-culture PBMCs were harvested and restimulated with either IL-12/ IL-15 for 16 h for assessment of IFN-γ production (Fig. 6.4 C) or with target cells for 4 h for evaluation of degranulation (Fig. 6.4 D). The percentage of IFN-γ producing NK cells from HCV-containing cultures was roughly twice as high as from NK cells from Huh6 or Huh6 cure cultures (44% +/-6% vs. 23% +/- 3% (Huh6) or 26% +/- 3% (Huh6 cure)) (Fig. 6.4 C).

However, degranulation of NK cells, as measured by the appearance of CD107a on the plasma membrane, which is normally on the membrane of cytotoxic granules, was similar in NK cells from all three Huh6 co-cultures against a standard target cell line (K562), but also against Huh6 cells with or without replicon. Of note, degranulation of NK cells, also from "PBMC only" conditions, against the Huh6 cell line war per se at a very high level. In fact, similar degranulation against Huh6 and the classical NK cell target K562 were observed. Furthermore NK cells from all co-cultures showed high background degranulation of around 40% even in the absence of target cell stimulation, whereas NK cells that were cultured within PBMCs without hepatocytes hardly showed degranulation without target cell stimulation (Fig. 6.4 D).

This "background" activation of NK cells in Huh6 and Huh6 cure co-cultures compared to the "PBMC only"-condition was also visible when analysing CD25 expression, proliferation and IFN-γ production, but in these conditions the presence of the virus furthermore enhanced NK cell activation (Fig. 6.4 A-C).

These results indicate that NK cells, in the first few days after encountering HCV-replicating hepatocytes, are activated, start to proliferate and specifically enhance their IFN-γ production, but not degranulation.

6.2.3 Characterisation of the CD25$^+$ NK cell subset

Looking at the histograms and graphs in figure 6.4 A-C it become visible that a distinct subset of NK cells expresses high amounts of CD25, has diluted CFSE and produces IFN-γ. Furthermore in all three read-outs the percentage of positive NK cells is almost

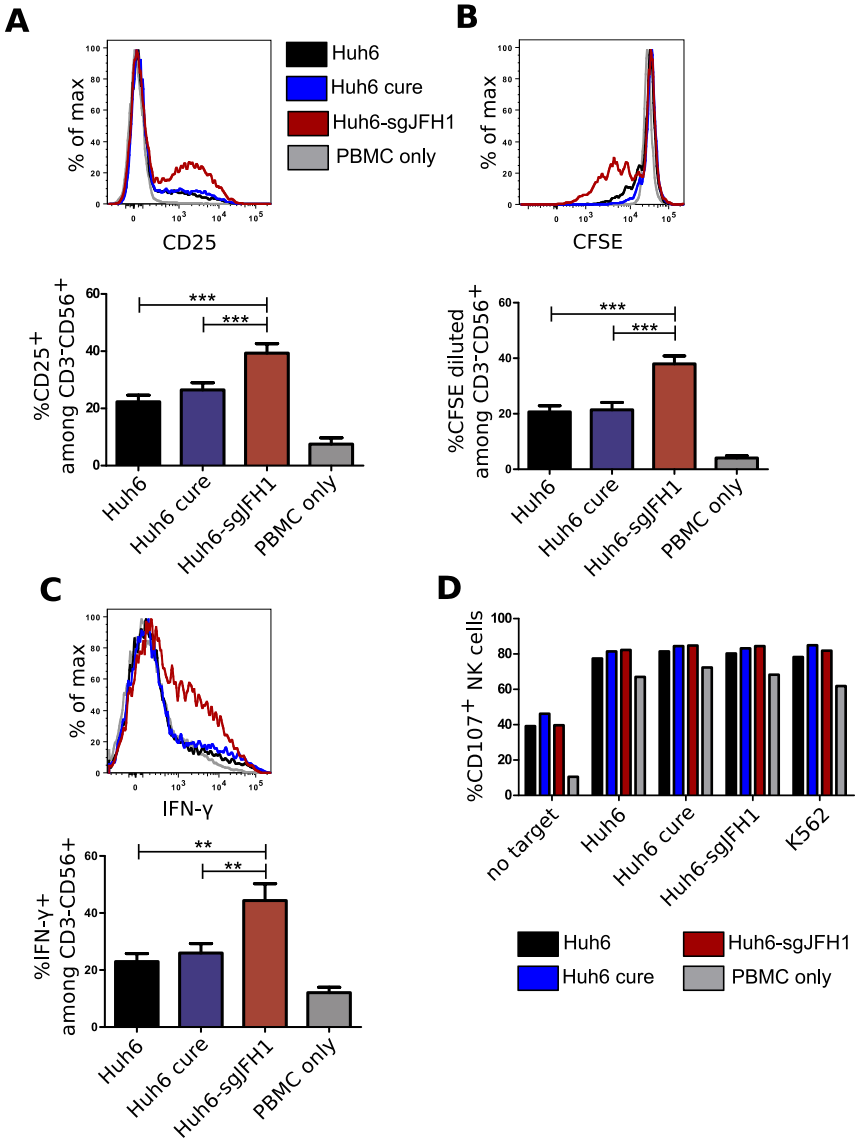

Fig. 6.4: CD25 expression, proliferation and IFN-γ production of NK cells are enhanced in Huh6-sgJFH1 co-cultures. Freshly isolated PBMCs from healthy donors were co-cultured with Huh6, Huh6 cure, Huh6-sgJFH1 cells or alone (PBMC only) for 6 days without the addition of cytokines. At the end of the co-culture, PBMCs were harvested and analysed by flow cytometry **A)-B)** or restimulated for functional analysis **C)-D)**. **A)** Representative histogram and summary for CD25 expression on NK cells after co-culture. n=29 **B)** CFSE dilution of NK cells at the end of co-culture for one representative donor and a summary of the percentage of cells with diluted CFSE for all donors is shown. PBMCs were stained with the proliferation tracking fluorescent dye CFSE before co-culture. n=18 **C)** IFN-γ production of NK cells was measured by intracellular flow cytometry staining. PBMCs at the end of the co-culture were restimulated for 16 h with 5 μg/ml IL-12 and 50 μg/ml IL-15 before IFN-γ analysis. n=10 **D)** PBMCs from indicated co-cultures were used in a 4 hour degranulation assay against Huh6, Huh6 cure, Huh6-sgJFH1, K562 cells or without target cells. Percentage of NK cells expressing CD107 after restimulation is shown for one representative out of two donors.
Gated on live, single $CD3^{-}CD56^{+}$ cells. Data depict mean + SEM, statistical significance was calculated using paired student's t test: ** $p < 0.01$, *** $p < 0.0001$

the same in all conditions. This promoted analysis of co-expression of these three markers, which is shown for one representative donor in figure 6.5 A. This reveals that indeed the same cells expressing CD25 are also the ones that have proliferated and produce IFN-γ. This multi-functional subset is already apparent after co-culture with parental and cured Huh6 cells but increases in size when the replicon is present. We further investigated this subset in respect to its expression of NKG2A, a receptor important for NK cell licensing. Analysis, shown for one representative donor in Fig. 6.5 B, illustrates that 13-14% of NK cells after Huh6 or Huh6 cure culture co-express CD25 and NKG2A and only 4-5% of CD25$^+$ cells are NKG2A$^-$. The double-positive cell population increases to 33% after Huh6-sgJFH1 culture, while the CD25$^+$ NKG2A$^-$ subset remains almost unchanged at 5.5%.

Our results demonstrate that a CD25$^+$ NKG2A$^+$ multi-functional NK cell subset is expanded after encountering HCV replicon-carrying Huh6 cells.

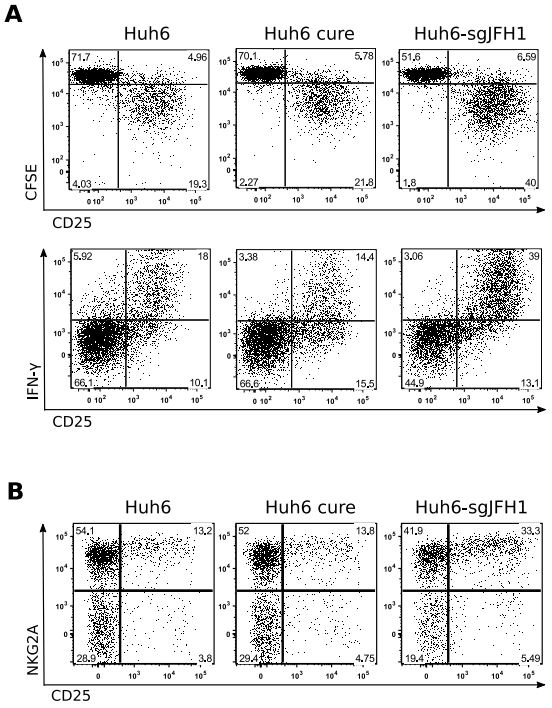

Fig. 6.5: The CD25$^+$ NK cells subset proliferates, produces IFN-γ-producing and expresses NKG2A Freshly isolated PBMCs from healthy donors were co-cultured with Huh6, Huh6 cure, Huh6-sgJFH1 cells for 6 days. At the end of the co-culture, PBMCs were harvested and analysed by flow cytometry. **A)** FACS dot plot of co-expression of CFSE (upper panel) or IFN-γ (lower panel) and CD25 on NK cells after indicated co-cultures. **B)** FACS dot plot of co-expression of NKG2A and CD25 on NK cells after indicated co-cultures.
One representative donor out of 5 is shown. Gated on live, single CD3$^-$CD56$^+$ cells.

6.3 Role of cytokines in the HCV-dependent activation of NK cells

6.3.1 Analysis and evaluation of cytokines in co-culture supernatants

Having observed phenotypical and functional changes of NK cells due to the presence of sg-JFH1 replicon, we were further interested to investigate the mechanisms through which these changes occur. Cytokines are important in shaping and regulating immune responses and many have been implicated in HCV infection.

Therefore we determined the concentration of pro- and anti-inflammatory cytokines IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A, GM-CSF, TNF-α and IFN-α in the co-culture supernatants by MACSPlex analysis and IL-18 by ELISA. IFN-α, although being one of the most studies cytokines in response to HCV infection and in the context of therapy, could not be detected in our co-culture supernatants. Also undetected, or detect at extremely low levels were IL-4, IL-5, IL-9, IL-10, IL-12p70, IL-17A, IL-18 and TNF-α (Fig. 6.6 C and data not shown). IL-6 and GM-CSF were present in the co-culture supernatants and furthermore specifically increased in the Huh6-sgJFH1 condition compared to Huh6 and Huh6 cure (Fig. 6.6 A-B, left column).

To investigate the importance of IL-6 and GM-CSF for the observed increase in $CD25^+$ NK cells in our co-culture system, the cytokines were neutralised by specific antibodies throughout the duration of the co-culture. The percentage of $CD25^+$ NK cells after 6 days of co-culture with either Huh6, Huh6 cure or Huh6-sgJFH1 was unchanged between isotype control and αIL-6 or αGM-CSF treated cultures (Fig. 6.6 A-B, right panel). A significant increase in $CD25^+$ NK cells between Huh6 / Huh6 cure and Huh6-sgJFH1 cultures was observed in both isotype and neutralising conditions.

IL-18 produced by monocytes was shown to increase NK cell function after encounter of JFH1-replicon carrying Huh7 cells in a similar co-cultures system (*122*), which prompted us to investigate its role in our system. However, in ELISA measurements of IL-18 in supernatants we did not detect IL-18 in Huh6 and Huh6 cure conditions and only one of three donors from the Huh6-sgJFH1 cultures showed minimal levels of IL-18, while it was undetectable in supernatants of the other two donors. In line with this finding, neutralisation of IL-18 with IL-18 binding protein (IL-18BP) had no effect on the percentage of $CD25^+$ NK cells (Fig. 6.6 C).

Many of the investigated pro- and anti-inflammatory cytokines were not or only at very low levels in co-culture supernatants and IL-6 and GM-CSF, although specifically elevated in Huh6-sgJFH1 co-cultures have no effect on the percentage of $CD25^+$ NK cells. We could also not confirm the previously published production of IL-18 in supernatants of Huh7-JFH1 co-cultures with PBMCs ((*122*)) and neutralisation of IL-18 did not affect $CD25^+$ NK cell percentages.

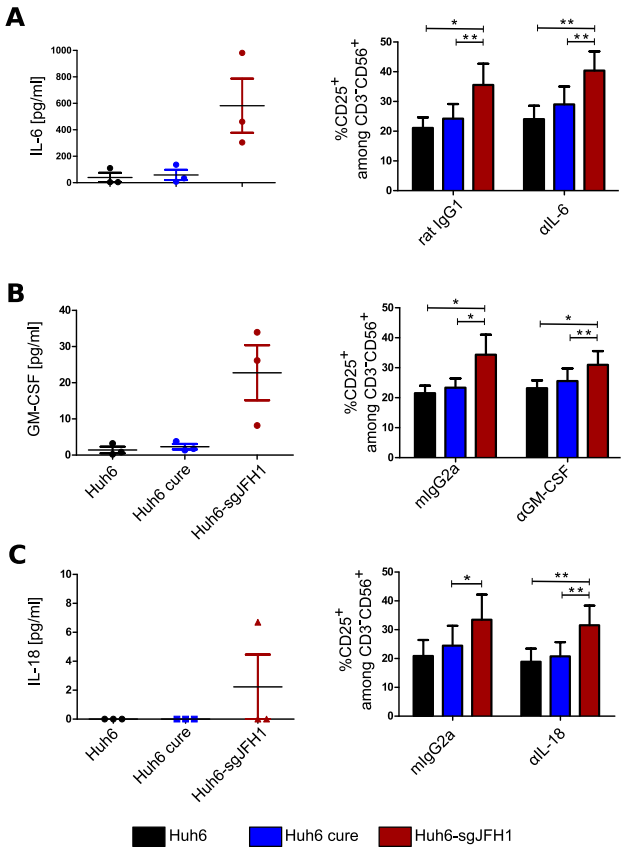

Fig. 6.6: No effect of IL-6, GM-CSF and IL-18 neutralisation on NK CD25 expression A)-C) left panel: Cytokine measurement by MACSplex **A)-B)** or ELISA **C)** of supernatants from 6 day co-culture of PBMCs with indicated Huh6 cell lines. n=3. **A)-C) right panel:** CD25 expression on NK cells after different PBMC - Huh6 co-cultures in the presence of isotype control or neutralising antibodies/proteins. **A)** Cultures were supplemented with 5 μg/ml neutralising αIL-6 or control rat IgG1 antibody. n=8 **B)** Cultures were supplemented with 7.5 μg/ml αGM-CSF of mIgG2a antibody. n=6 **C)** Cultures were supplemented with 2.5 μg/ml IL18BP or mIgG2a isotype control. n=7
For flow cytometric analysis cells were gated on live, single $CD3^-CD56^+$ cells. Data depict mean + SEM, statistical significance was calculated using paired student's t test: * $p < 0.05$ ** $p < 0.01$

6.3.2 Role of IL-2 in the activation of NK cells in Replicon co-cultures

Although IL-6 and GM-CSF were specifically increased in supernatants of PBMC - Huh6-sgJFH1 co-cultures, neutralisation of neither had an impact on NK cell activation. Next, we investigated IL-2, as one of the few receptors that was changed on NK cells after Huh6-sgJFH1 co-culture compared to Huh6 or Huh6 cured, was CD25, the α-chain of the trimeric IL-2 receptor complex, mediating high affinity binding for its ligand IL-2. We neutralised IL-2 by addition of neutralising antibodies. While in the isotype treated control CD25 levels on NK cells significantly increased on average from around 25% or 30% in Huh6 and Huh6 cure conditions, respectively to 40% in Huh6-sgJFH1 conditions, CD25 upregulation was greatly reduced to around 5% after IL-2 neutralization in all three conditions (Fig. 6.7 A)

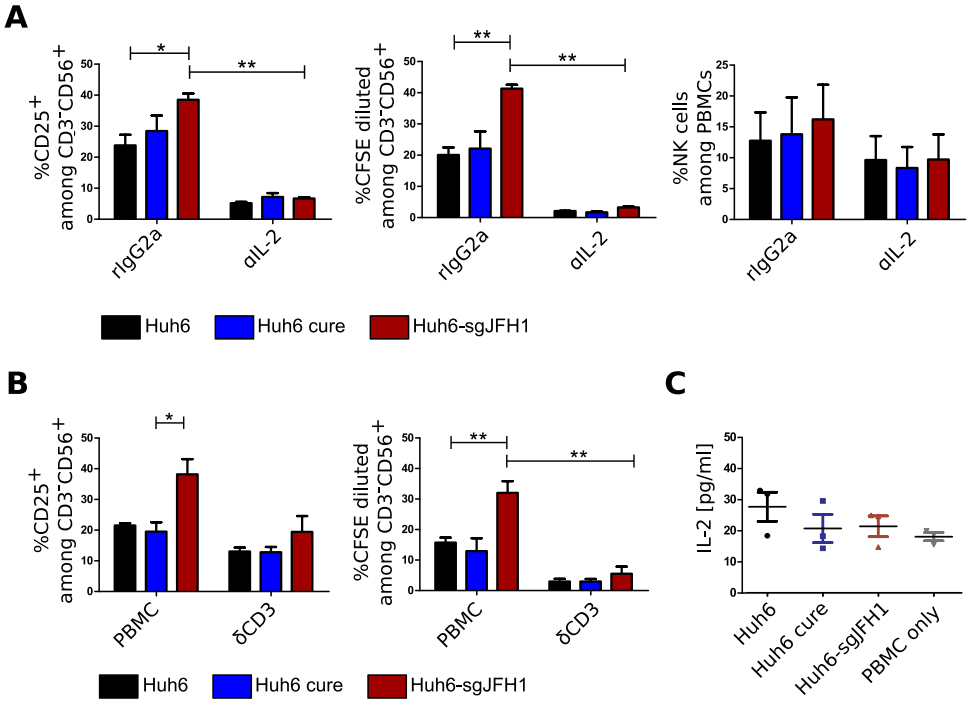

Fig. 6.7: IL-2 is important for NK cell activation in replicon co-cultures. Freshly isolated PBMCs from healthy donors were co-cultured with Huh6, Huh6 cure, Huh6-sgJFH1 cells for 6 days. **A)** Flow cytometric analysis of CD25 expression and CFSE dilution of NK cells and percentage of NK cells among all PBMCs after indicated PBMC - Huh6 co-cultures. Cultures were supplemented with 20 μg/ml neutralising αIL-2 or control rat IgG2a antibody. n=3 **B)** Flow cytometric analysis of CD25 expression and CFSE dilution of NK cells. Huh6 cell lines were either cultured with complete PBMCs or with PBMC MACS-depleted of $CD3^+$ cells (δCD3). n=3 **C)** MACSplex analysis of IL-2 from the indicated co-culture supernatants. n=3
For flow cytometric analysis cells were gated on live, single $CD3^-CD56^+$ cells. Data depict mean + SEM, statistical significance was calculated using paired student's t test: * $p < 0.05$ ** $p < 0.01$

The same pattern was observed for proliferation of NK cells, as measured by CFSE dilution. Neutralisation of IL-2 completely abrogated proliferation in all conditions, while in the isotype control again the doubling of the percentage of proliferated cells between Huh6/Huh6 cure and Huh6-sgJFH1 conditions from around 20% to 40% is observed.

The neutralisation of IL-2 affected not only the virus-induced upregulation of CD25 and NK cells proliferation, but also the observed "background" activation by the cancer cell line Huh6. This global effect on NK cell activation is not due to reduced survival of NK cells, because IL-2 neutralisation had only minor effect on the survival of NK cells over the course of the 6 day co-culture. The percentage of NK cells among whole PBMCs was similar in isotype and αIL-2 treated cultures and the number of 7-AAD$^+$ NK cells also remained unchanged (Fig. 6.7 and data not shown).

As the major source for IL-2 are typically T cells, we further investigated the role of T cells for NK activation in our system by depleting them from PBMCs via MACS (δCD3) (Fig. 6.7 B). Depletion of CD3$^+$ cells showed a minor reduction of the percentage of CD25$^+$ NK cells in Huh6 and Huh6 cure cultures and a stronger effect in the replicon cultures, although results did not reach significance with the small sample size yet. The reduction of proliferation after T cell depletion was more pronounced and could almost be completely abrogated in all three conditions. Of note CD3$^+$ cell depletion was only between 80%-85% in the tested donors, providing a possible explanation why results are not as pronounced as with IL-2 depletion (Fig. 6.7 B)

IL-2 is a necessary factor for the virus-induced and virus-independent acquisition of CD25 and proliferation of NK cells. However, it cannot be the decisive factor for the observed phenotypic and functional changes of NK cells in HCV-replicating cultures, as the levels of IL-2 in the supernatants of all culture conditions are comparable (Fig. 6.7 C). Levels of IL-2 in supernatants are also similar to Huh6 / Huh6 and Huh6-sgJFH1 conditions when PBMCs are cultured without hepatocytes, suggesting that T cells, or any other IL-2 producing cells, are not specifically activated by the hepatocytes due to e.g. possible allo-recognition or even virus-recognition.

6.4 Monocytes in the HCV-induced activation of NK cells

6.4.1 Requirement of monocytes for HCV-dependent NK cell activation

Our co-culture system is comprised of complete PBMCs, including NK cells, T cells, B cells, monocytes and DCs and hepatocytes. We have shown that T cells are important for the activation of NK cells in our system, most likely because of their production of IL-2. But neutralisation of IL-2 and depletion of T cells effected NK CD25 levels and proliferation in all conditions, not only the virus-dependent NK activation. To further dissect the possible mechanisms of virus-dependent NK cell activation we studied the importance of other cell types within PBMCs.

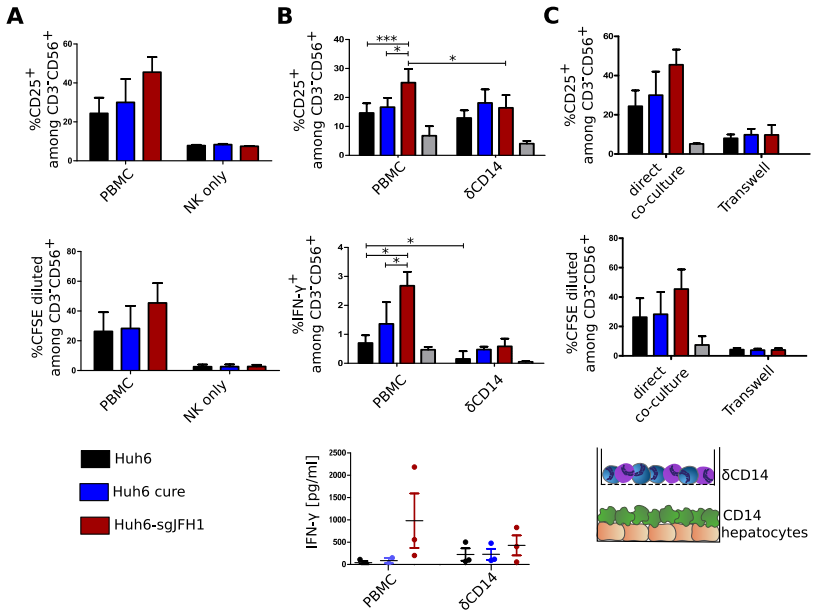

Fig. 6.8: CD14$^+$ cells are necessary for NK cell activation in co-cultures. A) Flow cytometric analysis of CD25 expression and CFSE dilution of NK cells on day 6 after indicated co-cultures. Huh6, Huh6 cure or Huh6-sgJFH1 cells were either cultured with complete PBMCs or with NK cells only. NK were isolated negatively via MACS. n=2 **B)** Flow cytometric analysis of CD25 (n=11) and IFN-γ (n=3) expression of NK cells on day 6 after indicated co-cultures and MACSplex measurement of IFN-γ (n=3) from co-culture supernatants. The Huh6 cell lines were either cultured with complete PBMCs or with PBMC depleted of CD14$^+$ cell via MACS (δCD14). **C)** PBMCs were either cultured directly with the indicated Huh6 cell lines or in a Transwell set-up for 6 days before flow cytometric analysis of CD25 expression and CFSE dilution of NK cells. For the Transwell set-up the different Huh6 lines were cultured together with MACS-isolated CD14$^+$ cells in the bottom and MACS-depleted of CD14 cells (δCD14) added in the upper chamber. n=2
Gated on live, single CD3$^-$CD56$^+$ cells. Data depict mean + SEM, statistical significance was calculated using paired student's t test: * $p < 0.05$, *** $p < 0.0001$

First of all, isolated NK cells were used in the co-culture experiments, but as expected, also due to the absence of T cells, no increase in $CD25^+$ NK cells and no proliferation was observed in any of conditions (Fig. 6.8 A). Even when we supplemented the co-cultures of isolated NK cells with 20 IU/ml IL-2 to compensate for the missing T cells, also no virus-dependent CD25 upregulation was observed (data not shown), indicating that factors from other cells within the PBMCs, besides IL-2, are important for the activation of NK cells.

The importance of monocytes for NK cell activation and function has been shown for HCV and other viral infections (*120*)(*122*)(*63*), which prompted us to investigate their role in our system.

$CD14^+$ monocytes were depleted from PBMCs by MACS positive selection and the remaining cells (δCD14) used for co-culture experiments. Depletion of $CD14^+$ cells specifically affected the percentage of $CD25^+$ NK cells in Huh6-sgJFH1 cultures, whereas percentages remained almost unchanged in Huh6 and Huh6 cure conditions (Fig. 6.8 B). IFN-γ production, without restimulation, was also reduced in δCD14 cultures, but also in the control conditions. Notably, percentages of IFN-γ producing NK cells were very small and differences are only minor.

Furthermore also soluble IFN-γ levels in supernatants were increased in Huh6-sgJFH1 - PBMC cultures compared to Huh6/Huh6 cure and this upregulation was greatly reduced in Huh6-sgJFH1 - δCD14 cultures (Fig. 6.8 B).

To dig deeper into the mechanisms of monocyte - NK cell interaction we performed transwell experiments to obtain information whether this cross-talk is mediated by soluble or cell-bound factors. We used hepatocytes together with isolated $CD14^+$ cells in the lower chamber. This ensured a possible activation of the monocytes by the virus, because for DCs it has been shown that their activation by HCV replicons is dependent on short-range exosomal transfer of viral RNA (*119*). In the upper chamber $CD14^+$ cell depleted PBMCs were cultured (δCD14) (Fig. 6.8 C).

Analysis of CD25 expression and proliferation of NK cells revealed that in the transwell setting both were almost completely abrogated. The "background" activation of NK cells was likely gone because the contact between the hepatoblastoma cells was missing and the virus-dependent activation was maybe gone because monocytes and NK cells require direct cell-to-cell contact. Another possibility would be that NK cells require stimuli by the monocytes as well as direct contact to the infected hepatocytes (Fig. 6.8 C).

Our experiments show, that $CD14^+$ monocytes are pivotal for the virus-dependent activation and function of NK cells in co-culture systems of PBMCs and HCV-replicon carrying hepatocytes and that NK cell - monocyte cross-talk is likely cell-cell-contact dependent.

6.4.2 Phenotype of $CD14^+$ cells in HCV co-cultures

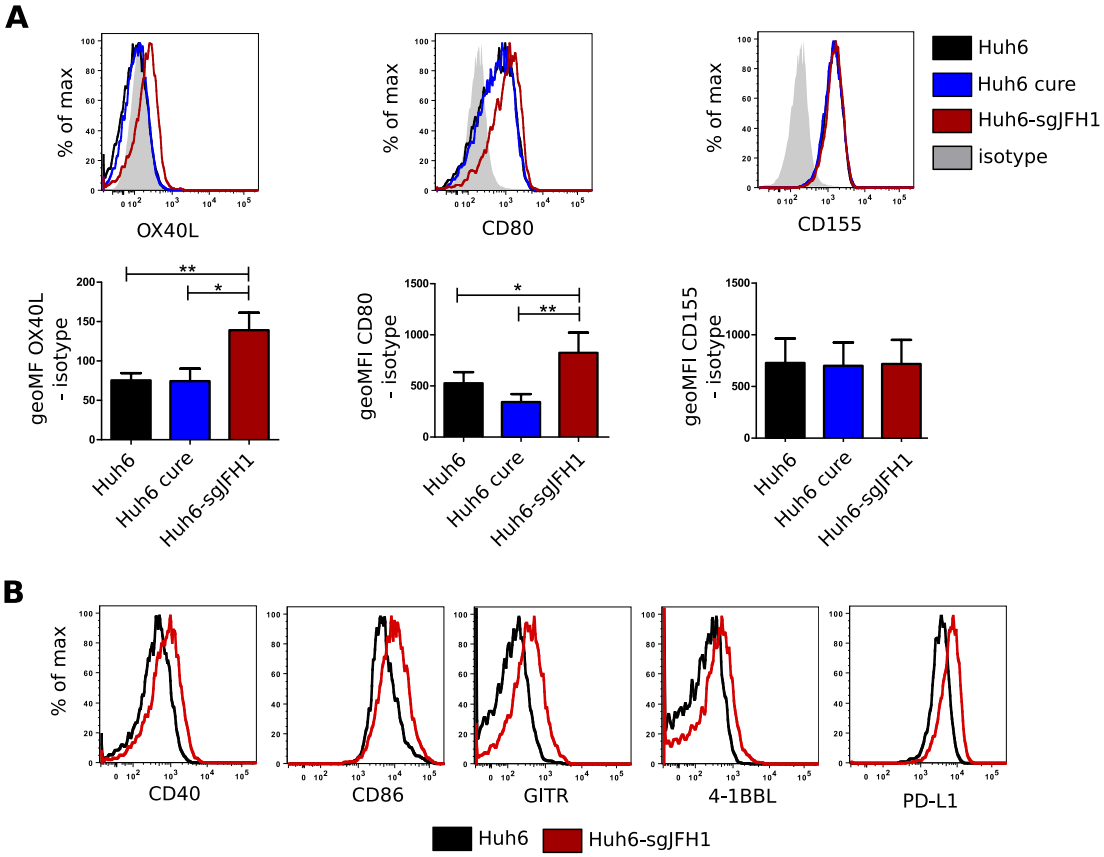

Fig. 6.9: Phenotype of $CD14^+$ cells in HCV^+-co-cultures. PBMCs were co-cultured with Huh6, Huh6 cure or Huh6-sgJFH1 cell lines for 48-60 hours before flow cytometric analysis. **A)** Flow cytometric histograms and summaries of all donors for OX40L, CD80 and CD155 expression on $CD14^+$ cells. n=6-9 **B)** Exemplary staining of indicated monocyte activation markers
Gated on monocyte gate, live, single, $CD14^+$, Data depict mean + SEM, statistical significance was calculated using paired student's t test: * $p < 0.05$, ** $p < 0.01$

Having established the importance of monocytes for the activation of NK cells in our system and several clues that this activation is based on direct cell-cell-contact, we

further investigated the monocytes from our co-cultures in regard to activation markers and potential interaction partners with NK cells.

Figure 6.9 shows that monocytes upregulated several general monocyte activation markers but also molecules which are important for a possible NK cross-talk.

One possible interaction partner with NK cells is the TNFSF member OX40L, whose expression level was significantly higher after 60 h PBMC- Huh6-sgJFH1 culture. In fact, in the control conditions expression of OX40L was virtually absent (Fig. 6.9 A). Further members of the TNFSF and TNFRSF, like 4-1BBL and GITR are also induced after virus-replicating cultures (Fig. 6.9 B), while other NK interaction partners, such as DNAM-ligand CD155, were expressed on monocytes, but remained unchanged after the different co-cultures (Fig. 6.9 A).

The T-cell co-stimulatory molecule CD80 (B7-1) is usually not expressed on naive $CD14^+$ cells but induced upon activation (*143*). Figure 6.9 shows in an representative histogram of one donor and a summary of all analysed donors that CD80 was induced on monocytes in all conditions, but induction was highest in virus-containing cultures. Other monocyte activation markers such as CD40 and CD86 were also induced after Huh6-sgJFH1 cultures (Fig. 6.9).

6.5 OX40 expression on NK cells

The upregulation of OX40L on monocytes attracted our attention, because in our initial NK receptor screen (Fig. 6.3), besides CD25, we also found OX40, the receptor for OX40L, higher expressed on NK cells after cultures containing HCV replicons. We could confirm this upregulation of OX40 on NK cells on a percentage and MFI level in more donors and show that OX40 is significantly higher on NK cells after 6 days of Huh6-sgJFH1 cultures, than after Huh6 or Huh6 cure cultures (Fig. 6.10 A). On freshly isolated NK cells, OX40 expression was virtually absent and it was only induced after culture (data not shown).

Furthermore when we analysed OX40 expression on the $CD25^+$ and $CD25^-$ NK cell subsets, we found that OX40 was almost exclusively expressed on the $CD25^+$, further substantiating a role in the activation of this on subset that also proliferated and produced IFN-γ (Fig. 6.10 B).

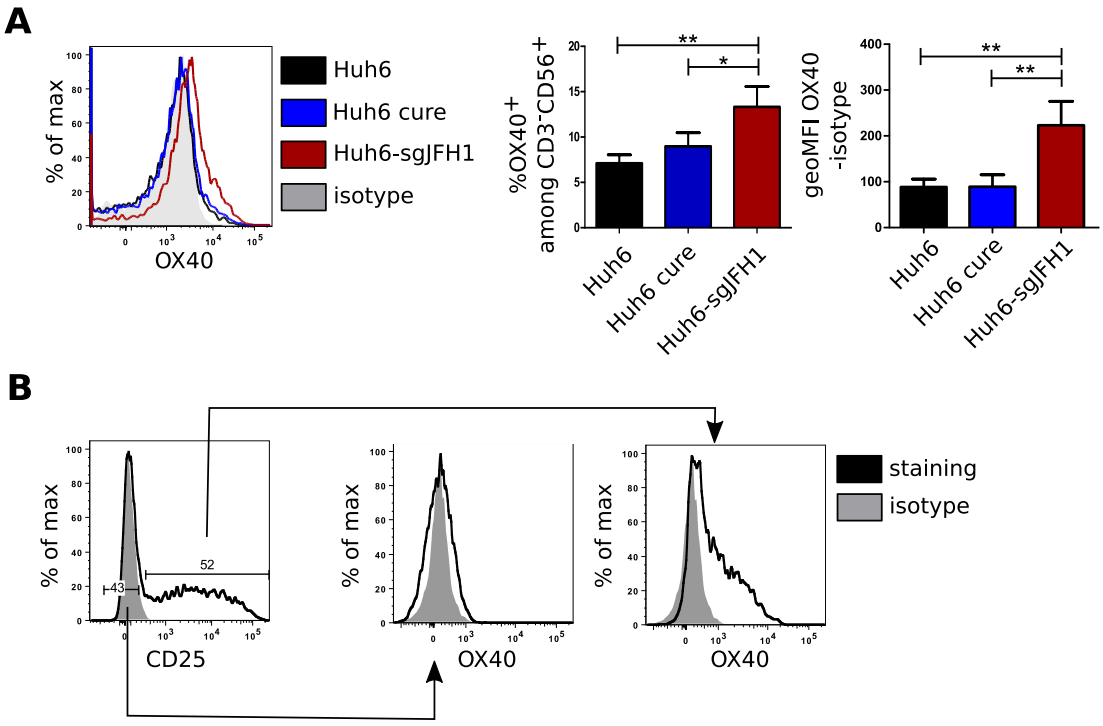

Fig. 6.10: OX40 expression on NK cells in HCV$^+$-co-cultures. PBMCs were co-cultured with Huh6, Huh6 cure or Huh6-sgJFH1 cell lines for 6 days and then analysed via flow cytometry. **A)** Representative histogram of OX40 expression by NK cells and summaries of all donors for the percentage of OX40$^+$ NK cells and the geoMFI of OX40 expression on all NK cells (n=10-13). **B)** Exemplary histograms of the OX40 expression on CD25$^+$ and CD25$^-$ NK cells after co-culture.
Gated on live, single CD3$^-$CD56$^+$ cells. Data depict mean + SEM, statistical significance was calculated using paired student's t test: * $p < 0.05$, ** $p < 0.01$

6.6 Relevance of the OX40 - OX40L interaction

6.6.1 Blocking of OX40L during co-cultures

After observing that OX40 was upregulated on NK cells and OX40L on monocytes specifically after Huh6-sgJFH1 cultures, we sought to further investigate this interaction. Blocking of OX40L by monoclonal antibody Oxelumab led to a reduction of CD25$^+$ NK cells in Huh6-sgJFH1 conditions but also, although to a lesser yet significant extent in Huh6 and Huh6 cure conditions. Blocking of OX40L by a different αOX40L antibody (clone MM0505-8S23) showed the same pattern in an initial experiment with one donor. CD25 levels on NK cells from the Huh6-sgJFH1 conditions could even be completely brought to the level observed in Huh6 conditions with this antibody

(Fig. 6.11 A). The percentage of CFSE diluted NK cells was significantly reduced upon blocking of OX40L with Oxelumab. The significant upregulation between control and replicon conditions seen in hIgG treated cultures was thereby greatly reduced and no longer significant in the Oxelumab treated cultures. As for CD25 expression, Oxelumab treatment also slightly reduced CD25 expression in control cultures. This observation was not seen when using the MM0505-8S23 antibody. Here OX40L blocking completely abrogated virus induced proliferation, bringing it to levels similar to uninfected controls, while having almost no effect on the Huh6 and Huh6 cultures. Of note, the MM0505-8S23 antibody was only used in one initial donor (Fig. 6.11 B).

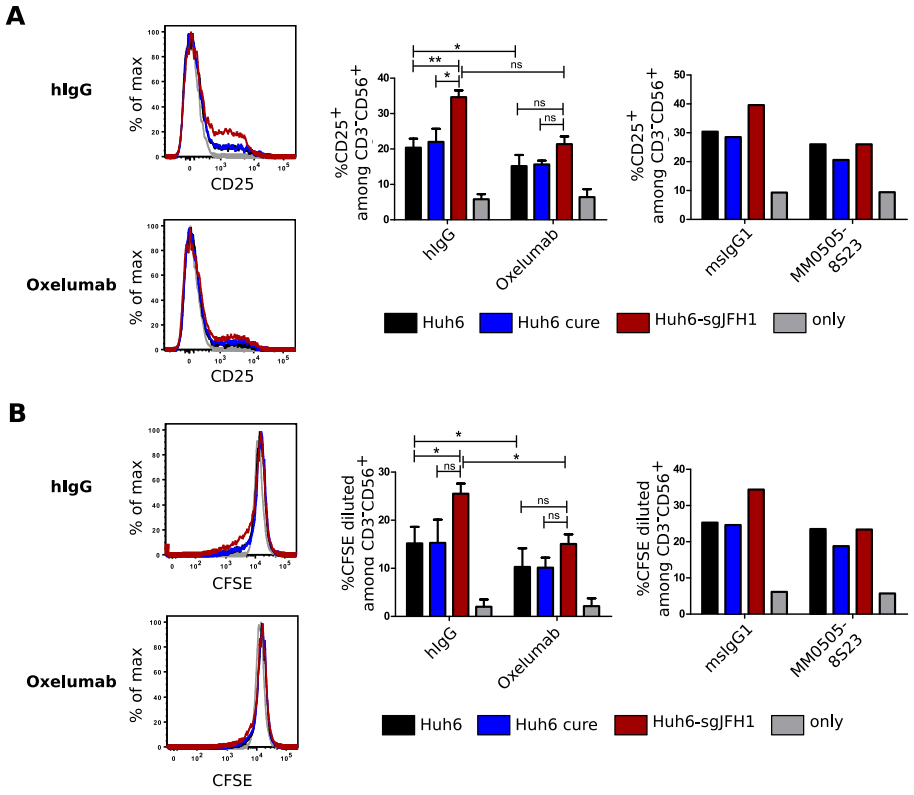

Fig. 6.11: Blocking of OX40L in PBMC - Huh6-sgJFH1 co-cultures inhibits upregulation of CD25 on NK cells and their proliferation. PBMCs were co-cultured with Huh6, Huh6 cure or Huh6-sgJFH1 cell lines for 6 days in the presence of OX40L blocking antibodies (either Oxelumab or MM0505-8S23) or isotype controls and then analysed by flow cytometry. **A)** Exemplary histograms and summary of CD25 expression on NK cells from the indicated co-cultures. **B)** Exemplary histograms and summary of CFSE dilution of NK cells from the indicated co-cultures.
Gated on live, single $CD3^{-}CD56^{+}$ cells. Oxelumab n= 3, MM0505-8S23 n=1. Data depict mean + SEM, statistical significance was calculated using paired student's t test: * $p < 0.05$, ** $p < 0.01$

6.6.2 OX40L expression on other cells within co-cultures

The observation that blocking of OX40L also to a small extent reduced CD25 levels and proliferation of NK cells in the uninfected co-cultures, led us to investigate the expression of OX40L on other cells within the co-culture system. And indeed, the Huh6, Huh6 cure and Huh6-sgJFH1 cell lines express OX40L on their surface, but all to the same degree (Fig. 6.12 A). Expression of OX40L on hepatocytes could be confirmed by immunohistochemistry staining of primary hepatocytes in liver biopsy samples of chronic HCV patients (Fig. 6.12 B). Other cells, besides hepatocytes seem to also stain positive for OX40L, but their exact nature has yet to be determined. No expression of OX40L was detected within the lymphocyte gate at day 6 in any co-culture conditions (Fig. 6.12 C).

6.7 HCV-specificity of NK cell activation

Finally we wanted to address whether this activation of NK cells via OX40L on monocytes that we observed in our PBMC - Huh6-sgJFH1 co-cultures was specific for HCV and whether it was indeed due to recognition of the virus.

To address the first point we made use of two other viral replicon systems. One is another HCV replicon system which uses Huh7 cells that carry the 9-13 replicon, which is of a different HCV genotype than JFH1. The 9-13 replicon is genotype 1b and JFH1 is genotype 2a. The other one is a Dengue virus (DENV) replicon that was also electroporated into Huh6 cells. DENV is located in the same family as HCV, the flaviviridae family, but under a different genus. HCV is from the hepacivirus genus, while DENV, like many other vector-transmitted viruses (Zika virus, Yellow fever virus or West Nile virus) is within the flavivirus genus.

We again co-cultured Huh6, Huh6 cure, Huh6-sgJFH1 as well as the Huh6-DENV, Huh7 and Huh7 9-13 cell lines together with PBMCs for 6 days and then analysed CD25 expression, proliferation and IFN-γ production after IL-12/15 restimulation (Fig. 6.13). The Huh6-DENV cultures were not significantly different to the Huh6 cultures in any of the read-outs, suggesting that the presence of the HCV-related DENV replicon did not activate proliferation and IFN-γ production of NK cells in this setting.

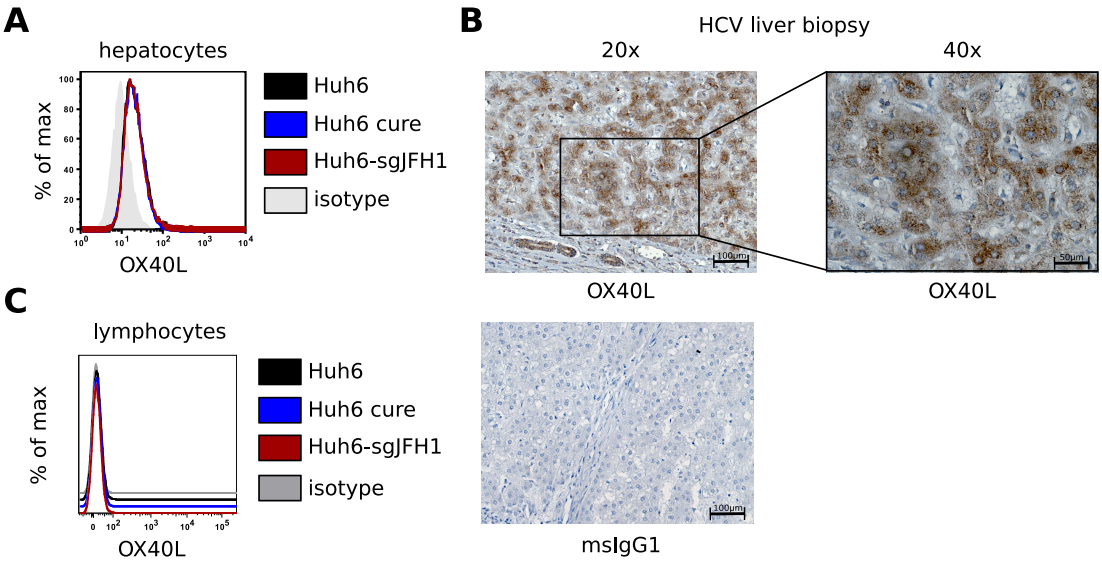

Fig. 6.12: OX40L is expressed on hepatocytes, but not on lymphocytes after co-cultures. **A)** Histogram of flow cytometry staining of OX40L on Huh6, Huh6 cure and Huh6-sgJFH1 cells. **B)** Immunohistochemistry staining with α-OX40L or isotype antibody on liver biopsy from a chronic HCV patient. **C)** Histogram of flow cytometry staining of OX40L lymphocytes after indicated co-cultures. PBMCs were co-cultured for 6 days with indicated cell lines before flow cytometry analysis. Cell were gated on live, single, lymphocytes.

In the co-cultures with Huh7 9-13 replicon cells, NK cells showed significantly higher CD25 expression, although this was generally at a much lower level in all Huh7 than in the Huh6 cultures. There was also a trend towards higher proliferation in the Huh7 9-13, than in the Huh7 cultures and finally IFN-γ production was strongly increased in the Huh7 9-13 compared to the parental cultures. This induction of IFN-γ was slightly more pronounced than in the Huh6 system (Fig. 6.13).

To address the second point, we wanted to block recognition of the viral RNA to check if the observed activation is in fact due to virus recognition. Monocytes mainly recognise viral RNA via their endosomal TLRs. These can be inhibited using Bafilomycin A which blocks acidification of endosomes. Unfortunately PBMCs did not survive 6 day treatment with Bafilomycin A at concentrations necessary to inhibit TLRs (data not shown). Because it was shown that viral replicon RNA can be transported out of the cell and activate DCs via exosomes, we used GW4869, an exosome inhibitor in co-culture experiments, followed by assessment of CD25 and CFSE dilution of NK cells (Fig. 6.14). There is a small trend, that the blocking of exosomes impairs CD25 upregulation and proliferation in the Huh6-sgJFH1 co-cultures, but not in the control conditions. The differences are not significant, but sample size is also still very small.

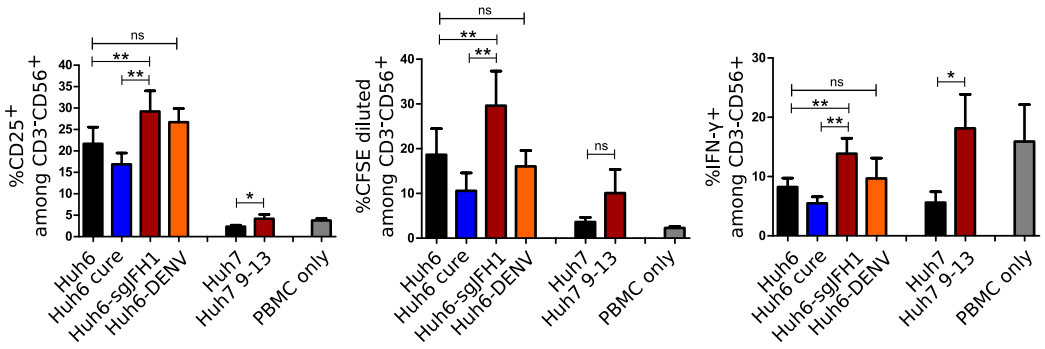

Fig. 6.13: Different HCV replicon systems, but not DENV replicons can activate NK cells. PBMCs were co-cultured with Huh6, Huh6 cure, Huh6-sgJFH1, Huh6-DENV, Huh7 or Huh7 9-13 cell lines for 6 days and then analysed by flow cytometry. Cells were labelled with CFSE on day 0. For IFN-γ read-out, PBMCs were restimulated for 16 h with 5 μg/ml IL-12 and 50 μg/ml IL-15 at the end of the co-culture. n=3 Gated on live, single $CD3^{-}CD56^{+}$ lymphocytes. Data depict mean + SEM, statistical significance was calculated using paired student's t test: * $p < 0.05$, ** $p < 0.01$

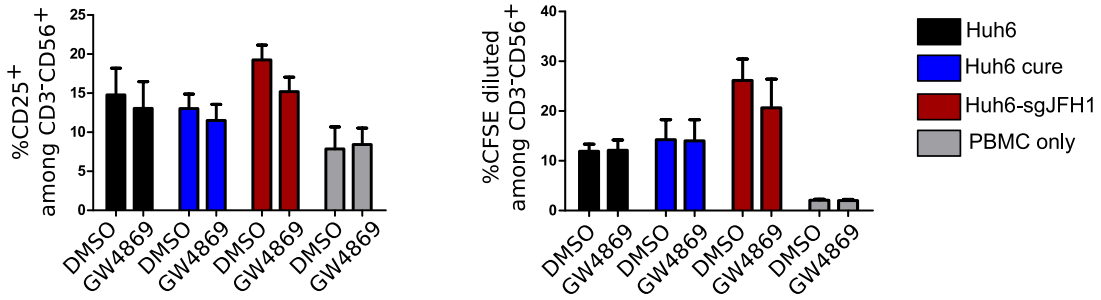

Fig. 6.14: Assessing importance of exosome release on NK cell activation. PBMCs were co-cultured with Huh6, Huh6 cure, Huh6-sgJFH1 cell lines for 6 days in the presence of 10 μM GW4869 or equivalent amounts of DMSO. Cells were labelled with CFSE on day 0. n=3
Gated on live, single $CD3^{-}CD56^{+}$ lymphocytes. Data depict mean + SEM.

Our data show that the observed NK cell activation is seen in different cell culture models of HCV, but not with the related DENV and that blocking of exosome-mediated viral recognition might reduce NK cell activation.

Taken all our results together we could show that in HCV-replicon co-cultures with PBMCs an NK cell subset shows higher CD25 and OX40 expression and more proliferation and IFN-γ production. Monocytes, activated by the recognition of viral RNA, likely contained in exosomes, are crucial for the activation and IFN-γ production of NK cells. OX40L is upregulated on monocytes, which could stimulate NK cells via OX40. The OX40-OX40L interaction, as well as IL-2 sensing via CD25/CD122/CD132 leads to enhanced proliferation of NK cells.

7 Discussion

HCV infection and the consequent HCV-related liver diseases are among the biggest global health problems. Although virus prevalence has peaked in 1994 and is thought to further decline, the number of HCV-related liver diseases such as fibrosis, cirrhosis and ultimately cancer are still predicted to rise over the next decades, because those complications only manifest after several decades of infection (*144*). DAAs treatment can help reduce liver complications, but they are only available to a very small number of patients and the emergence of resistant virus strains is always a risk. So in spite of the highly effective drugs against HCV, further research is needed to help create effective vaccines and other treatment options. One possible way to achieve this is to better understand the natural immune response that leads to spontaneous viral clearance.

Previous studies have implicated a role for NK cells in the anti-HCV immune response, but mechanistic insights are sparse. Therefore the aim of this study was to decipher the role of NK cells in the immune response against HCV infected hepatocytes.

We show that NK cells in a co-culture of PBMCs and HCV-replicon carrying Huh6 liver cells increase the percentage of the high-affinity IL-2 receptor CD25 and the TNFSF-receptor OX40. Functionally they have a higher proliferation and IFN-γ production than NK cells from co-cultures without replicon. NK cell activation was dependent on IL-2, on OX40-OX40L interaction and on $CD14^+$ monocytes.

7.1 The Replicon Systems

To gain mechanistic insights into the interplay of NK cells, infected hepatocytes and other immune cells, we decided to use the *in vitro* HCV replicon system for our studies.

We tested three cell lines stably replicating the subgenomic JFH1 replicon: Huh7, Huh6 and HepaRG. Huh7-sgJFH1 had the highest viral RNA levels. HCV RNA levels

in Huh6-sgJFH1cell were around 20% lower than in Huh7-sgJFH1 cells and in HepaRG-sgJFH1 cells even more than 90% lower (data not shown).

Analysing expression of NK cell ligands on parental, cured and replicon-carrying Huh7, Huh6 and HepaRG cells, we did not observe any significant changes in any of the cell lines (Fig. 6.2 and data not shown). This is in accordance with Stegmann et al. who also did not observe changes in NK cell ligands after JFH1 infection of Huh7.5 cells (*109*). A possible explanation could be that due to the malignant transformation of the Huh7 and Huh6 cells, the mainly stress-induced NK cell ligands are already maximally induced. Indeed, we do observe expression of many NK cell ligands which are usually absent on healthy cells already on the parental cell lines (Fig. 6.2 and data not shown). For example NKG2D-ligands MIC-A and MIC-B were shown to be expressed in HCV-infected liver tissue, but not in healthy livers (*112*). In our cell lines however MIC proteins are already expressed in the parental lines (Fig. 6.2 and data not shown). For the HepaRG line, which is a hepatic precursor cell line that is very similar to normal non-transformed hepatocytes, viral replication might have been too low and they also, to some extent, already express activating NK cell ligands. Another possible explanation could be virus escape mechanisms that interfere with upregulation of NK cells ligands, which have already been described for NKG2D-ligands (*111*). Furthermore, many NK cell ligands still remain elusive. However, no alterations in NK cell phenotype or function were observed after co-culture of isolated NK cells with Huh6-sgJFH1 cells (Fig. 6.8) arguing against regulation of yet unknown NK cell ligands.

7.2 NK cell activation in replicon co-cultures

7.2.1 Phenotype of NK cells

Even though the replicon did not directly regulate NK cell ligands on the hepatocyte surface, NK cell phenotype was changed after several days of co-culture of PBMCs with replicon-carrying Huh6 cells.

A higher percentage of NK cells from the Huh6-sgJFH1, than from Huh6 or Huh6 cure, conditions expressed the high-affinity chain of the IL-2 receptor, CD25 and the TNFSF-receptor OX40 (Fig. 6.4 and 6.10). The induction of both CD25 and OX40 is also observed on antigen-specific T cells after restimulation with cognate antigen (*32*). But

the mechanism of this upregulation in our system, as well as on T cells, is still unclear. Because CD25 is expressed only at low levels in $CD56^{bright}$ NK cells and completely absent on $CD56^{dim}$ NK cells and OX40 is not expressed at all on freshly isolated NK cells, their expression must be actively induced, as opposed to e.g. outgrowth of a subset already expressing the receptors (*47*). Of note, the subset expressing high levels of CD25 and OX40 also proliferates more (Fig. 6.4), which can then further support the increase in percentage.

We have observed that expression levels of OX40 on NK cells correlate with levels of IL-2 present in the co-cultures. When co-cultures were supplemented with 100 U/ml IL-2, the overall levels of OX40 were higher than in co-cultures without exogenous IL-2 (compare OX40 levels in figure 6.3 (100 U/ml IL-2) and 6.10 (no added IL-2)). Also when the same donors were cultured with or without IL-2, OX40 was present on approximately 50% of NK cells in cultures with 100 U/ml IL-2 and only on around 5-10% on NK cells cultured without added IL-2 (data not shown). For T cells it has already been shown that IL-2 can induce and enhance the expression of OX40 and other TNFRSF members, although the primary OX40-inducing stimulus in T cell is TCR and CD28 or CD40 engagement (*30*) (*31*) (*145*). The induction of OX40 on NK cells in our co-culture system could hence be facilitated by IL-2. Although IL-2 levels are similar in all tested conditions (Fig. 6.7), OX40 induction could be higher in Huh6-sgJFH1 conditions because signalling of IL-2 is enhanced due to expression of CD25. It has been shown that cells with and without CD25 expression can proliferate equally under high IL-2 concentrations ($>$ 100 U/ml), but only CD25 expressing cells can proliferate under low IL-2 concentrations, such as in our system (only around 20 pg/ml) (*146*) (*147*). Thus, enhanced IL-2 signalling due to expression of CD25 in Huh6-sgJFH1 conditions could contribute to the enhanced OX40 expression on NK cells. However, blocking of OX40L-OX40 interaction also prevented CD25 upregulation, which would argue against a sequential regulation of CD25 and OX40. Furthermore in time-course experiments, OX40 and CD25 expression were increasing rather simultaneously (data not shown), arguing for a more complex transcriptional regulation.

CD25 upregulation on NK cells is observed in a number of situations, such as viral or bacterial infections, vaccination or cytokine treatment (*63*)(*147*)(*148*) (*49*)(*50*). In many of the above mentioned scenarios, the pro-inflammatory cytokines IL-12 and IL-18, which are often produced during viral or bacterial infections, are involved in the induction of CD25. Lee et al. even observed upregulation of CD25 only on a subset of NK cells after IL-12 stimulation, similar to our observations. However, neither IL-12p70

nor IL-18 could be detected by MACSplex or ELISA, respectively, in our co-culture supernatants (Fig. 6.6 C and data not shown) and neutralisation of IL-12 or IL-18 by neutralising agents did not affect CD25 levels on NK cells (Fig. 6.6 C and data not shown). Recently also IL-27, a member of the IL-12 cytokine family was shown to be able to induce CD25 (*149*) and to be expressed by macrophages after TLR stimulation (*150*). IL-27 could therefore be a candidate for the virus-dependent induction of CD25 in our system. Macrophages/Monocytes are a likely source of the HCV-dependent stimulus for upregulation of CD25 and OX40, as without them CD25 levels in control and sg-JFH1 conditions are similar (Fig. 6.8).

We also observe that neutralisation of IL-2 abrogates CD25 expression almost completely, showing that IL-2 itself is necessary for the expression of its receptor. But CD25 expression was abrogated in all conditions, with and without replicon, and IL-2 is present at equal levels in all conditions, indicating that IL-2 is not the virus-dependent stimulus that enhances CD25 expression specifically in the Huh6-sgJFH1 conditions (Fig. 6.7).

Further investigations are needed to determine the factors induced by the HCV replicon which trigger the upregulation of CD25 and OX40 on NK cells.

7.2.2 Donor-to-donor variability

Working with primary human material often results in high experimental donor-to-donor variability. We also observed that some donors had a strong induction of e.g. CD25 or IFN-γ in response to the replicon, while others showed little or no signs of activation. However, the majority of the donors' NK cells were activated by the replicon. In more than 50% of cases an increase in the percentage of CD25 of more than 1.5-fold between Huh6-sgJFH1 and Huh6 conditions was observed. The donor with the strongest activation had more then 6 times more $CD25^+$ NK cells in the Huh6-sgJFH1 than in the Huh6 culture.

Possible reasons for these differences are most likely manifold and include sex, age, genetic factors (such as SNPs), or a different KIR - MHC repertoire, just to name a few. Further influencing factors could be previous infections or co-infections (eg. HCMV, HSV, EBV) with other viruses or pathogens. The phenomenon that other infections influence the immune response to a new pathogen has been shown for T cells. Previously formed memory T cells can cross-react with similar peptides from a different

pathogen and therefore excert strong and fast immune responses even after a primary pathogen encounter (*151*). There could be a similar mechanism in NK cells. Infection with HCMV or treatment with a combination of IL-12/15/18, cytokines often induced during viral infection, induce so-called adaptive or memory-like NK cells. These NK cells have been shown to have long-lasting heightened effector potential (ADCC and IFN-γ production) to pathogen-unspecific stimuli (*152*)(*153*) (*154*).
We have observed that NK cells that show a strong virus-dependent activation are in most cases already more activated by encountering the parental Huh6 cancer cell line than NK cells from donors that are not or only slightly activated by the replicon (data not shown). This argues that NK cells from these donors are in general more easily activated and show stronger effector responses, which could be because of previous stimuli such as HCMV.

Because monocytes are essential for the activation of NK cells in our system, differences in their activation potential and virus recognition could also affect NK cells. It was, for example, previously shown that a SNP in TLR-7 affects the progression of HCV infection (*155*).

7.2.3 Function of NK cells

NK cells from PBMC - Huh6-sgJFH1 co-cultures showed higher IFN-γ production with and without IL-12/15 restimulation and stronger proliferation than NK cells from PBMC - Huh6 / Huh6 cure co-cultures. Degranulation, as measured by the appearance of CD107a on the NK cell plasma membrane, against standard NK cell target lines (K562) and the Huh6 cells with and without replicon was, however, similar in NK cells from all different co-culture conditions (Fig. 6.4).

Previous studies using similar PBMC co-culture systems, albeit with JFH1 infected Huh7.5 or Huh7-sgJFH1 cells and for only 24 hours, did also observe an increase in NK cell IFN-γ production (*120*)(*122*). Yet, Serti et al. additionally reported an increase in NK cell cytotoxicity, which is also often reported for NK cells isolated from the blood of chronic HCV patients (*156*)(*137*). Mechanistically the increase in NK cell cytotoxicity was attributed to altered IFN-α signalling in chronic HCV patients, showing preferential STAT1 over STAT4 phosphorylation, which polarises NK cell towards cytotoxicity instead of IFN-γ production (*137*). Of note, in the chronic phase this altered signalling rather contributes to liver injury than viral clearance and also in the replicon *in vitro*

system viral replication was mostly influenced by IFN-γ production and not by cytotoxicity. Possible reasons for the discrepancy to our observations are the source of PBMCs and the duration of our co-cultures. PBMCs isolated from buffy coats, rather than from fresh blood contain very low numbers, if any pDCs, the main producers of IFN-α. Additionally pDCs have a very short live span *in vitro*, which is maybe why we do not detect IFN-α in co-culture supernatants from day 6 and subsequently no increase in NK cell cytotoxicity.

Proliferation of NK cells could be almost entirely abolished in all conditions by neutralisation of IL-2 (Fig. 6.7), indicating that IL-2, independent of the virus, is essential for the proliferation of all NK cells. However, under limiting IL-2 concentration the higher CD25 expression in the replicon-containing conditions probably leads to a stronger proliferation in the Huh6-sgJFH1 conditions.
Blocking of OX40L-binding to OX40 also affected proliferation, although only slightly in the Huh6 and Huh6 cure conditions and to a greater extent in the Huh6-sgJFH1 co-cultures (Fig. 6.11), which suggests that the proliferation dependent on OX40 signals might be induced by the virus. The dependence on IL-2 and OX40 in regard to IFN-γ production of NK cells in our system has yet to be evaluated.

The influence of OX40 signalling on NK cell proliferation is in line with previous reports, in which treatment of late-stage cancer patients with an agonistic OX40 antibody led to higher proliferation of $CD4^+$, $CD8^+$ T cell and NK cells, but not Treg cells (*36*). The finding that the $CD25^+OX40^+$ subset of cells are the main producers of IFN-γ and show the strongest proliferation is also similar to findings on CD4 T cells. Zaunders et al. could show that a subset of human CD4 T cells expresses CD25 and OX40 after antigen-stimulation. Only the double-positive subset produced significant amounts of IFN-γ and it showed the strongest proliferation (*32*). In mouse T cells OX40 signalling was shown to be crucial for the control of persistent viral infections (lymphocytic choriomeningitis virus (LCMV)) by promoting T cell survival through the activation of NF-κB and Akt which subsequently upregulates anti-apoptotic Bcl-2 family members (*157*). But in mouse T cells, proliferation and function were not affected by OX40 signalling. The importance of OX40 and the interplay with IL-2R, TCR and CD28 signalling for survival, proliferation and IFN-γ production were also shown in mouse NKT cells, where signalling through OX40 is thought to increase important transcription factors such as nuclear factor of activated T-cells (NFAT), NF-κB and Tbet which enhance IL-2 and IFN-γ production (*158*).
There are several indications from human T cells and mouse T and NKT cells that

OX40 signalling is mediating survival, proliferation and IFN-γ production and that the CD25 and OX40 pathways interact with each other, supporting our findings. However, to our knowledge, OX40 signalling has not been addressed in NK cells.

7.2.4 Characterisation of the activated NK cell subset

We consistently observe that only a specific subset of NK cells expresses CD25, proliferates and produces high amounts of IFN-γ (Fig. 6.5 A). This subset is almost completely positive for NKG2A (Fig. 6.5 B) and expresses OX40 (Fig. 6.10 B). The exact reasons why this specific subset is activated in our co-cultures remains elusive.

One possible explanation could be the KIR repertoire and the KIR - MHC-I matching of NK cells and Huh6 cells. Because the expression of the inhibitory KIR receptors is stochastic, every NK cell can express varying KIR numbers (0-4) and alleles leading to a different inhibitory potential by the interaction with the MHC-I$^+$ Huh6 cells. HLA typing of the Huh6 cells revealed expression of all 6 possible MHC-I molecules, including two HLA-C alleles, both of the C1 type (data not shown). HLA-C1 and -C2 are distinguished by either expression of the amino acid asparagine or lysin on position 80 and they can bind to different KIRs (*159*). To address this question experimentally, further stainings of different KIRs and KIR typing of each donor needs to be performed.

Beside the direct inhibitory potential of the KIRs, the NK cell education and licensing, which is largely dependent on KIR and NKG2A could also play a role in their subsequent activation potential. The fact that the activated subset is almost completely NKG2A$^+$ points in this direction, but further investigation into the HLA and KIR haplotypes of the donors would be necessary to answer this question.

The finding that the responding subset expresses NKG2A could also indicate that the CD56^{bright} NK cells is the subset specifically responding in our co-cultures, because the CD56^{bright} NK cells are known to all express NKG2A, while only about half of the CD56^{dim} NK cells express this receptor (*17*). However, the separation of those two subsets in flow cytometric analyses after the co-cultures is in most cases impossible (data not shown). The observation that they produce higher amounts of cytokines, but cytotoxicity is not affected in our studies would also fit to the classical definition of those two subsets, in which the CD56^{bright} NK cells are the main producers of cytokines, while the CD56^{dim} subset primarily kills target cells.

Another possible explanation could be the distribution of other receptors that are responsible for the induction of CD25 or OX40 on this subset. The receptor for the before mentioned IL-27 that could be a potential candidate for the virus-dependent NK cell activation, because it is secreted by monocytes after TLR stimulation and can induce CD25 expression, is expressed at a higher level in the $CD56^{bright}$ NK cell subset (*160*). However, the activation of this specific NK cell subset is also, although to a lesser extent, seen in the control conditions without virus, arguing for a virus-independent mechanism, such as the KIR distribution or licensing status of NK cells or a coexistence of both virus-dependent and independent mechanisms.

A similar subset of cells expressing high levels of CD25 and OX40 and increased IFN-γ production was observed in $CD4^+$ T cells. Analysis of this subset revealed that those are antigen-specific memory $CD4^+$ cells. In fact, a clinical assay has been developed that uses the induction of CD25 and OX40 after antigen-stimulation to determine whether a patient has memory CD4 T cells for a specific pathogen (*32*). The "OX40 assay" was subsequently shown to be also valid HCV antigens (*33*). Furthermore OX40 is not only a marker of memory $CD4^+$ T cells, but is actively involved in the generation of $CD4^+$, as well as $CD8^+$ memory T cells, e.g. by diminishing activation-induced cell death in T cells after antigen priming (*161*).
Recently, adaptive or memory-like NK cells were described in mice and humans. Interestingly, CD25 was one of the markers that was increased on IL-12/15/18 stimulated NK cells and after HCMV infection. Both scenarios which generate those adaptive NK cells(*153*) (*63*). Furthermore, in mice, memory NK cells were specifically found to reside in the liver (*162*), however in humans initial studies are still controversial regarding memory phenotype of NK cells in the liver (*163*)(*164*).
In the context of these findings on memory T and adaptive NK cells it is tempting to speculate that the expanding subset in our co-cultures would be endowed with long-lasting enhanced effector functions.

Those adaptive NK cells have already been detected in chronic HCV patients, although only in the context of HCMV co-infection. An increase in the $NKG2C^+$ NK cell subset, which was described to posses those adaptive features, was seen in chronic HCV and HBV patients that were $HCMV^+$ and this subset was highly functional (*165*). More recently negativity for the adaptor protein FcεRIγ is used as a marker for adaptive NK cells. This FcεRIγ^- subset largely, but not completely overlaps with NKG2C expression and is expanded in $HCMV^+$ healthy subjects as well as in $HCMV^+$ chronic HCV patients. Furthermore HCV^+HCMV^+ patients who posses the FcεRIγ negative

NK subset show reduced liver enzymes and fibrosis stage (*166*).
Further evaluation of cell with potential long term effector functions, by e.g. evaluating FcεRIγ expression of the expanded subset in our cultures would therefore be a worthy endeavour.

7.3 Importance of CD14$^+$ cells for the activation of NK cells

The depletion of CD14$^+$ cells from PBMCs in our co-cultures prevented the induction of CD25 and IFN-γ on NK cells in Huh6-sgJFH1 conditions.

The ability of PAMP-stimulated monocytes or macrophages to activate NK cells and the importance of monocyte/macrophage - NK cell cross-talk has been shown for many types of viral, bacterial and parasitic infections, whereby the NK stimulation can be achieved either via cytokines or via cell-cell-contact (*60*).

IL-12 secreted by inflammatory monocytes in HCMV infection is for example important for the expansion of adaptive NK cells and the increase of CD25 on those NK cells (*63*).

In the liver, IL-12 and IL-18 produced by Kupffer cells after polyI:C treatment were shown to stimulate liver-resident NK cells to produce IFN-γ (*167*). For HCV infection NK activation dependent on accessory cells (monocytes and dendritic cells) was shown in several *in vitro* studies, including the results presented in this thesis. Serti et al., Zhang et al. and we unanimously report that monocytes are important for NK cell activation in PBMC co-cultures with JFH1 infected or replicon-carrying hepatocytes and that isolated NK cells in co-cultures are not activated (*120*) (*122*) (Fig. 6.8). In the report by Serti et al. it was shown that IL-18 produced by monocytes in JFH1 infected Huh7.5 cell co-cultures can activate NK cells to produce higher amounts of IFN-γ (*122*). However, this finding was not confirmed by Zhang et al. using the same infection system, neither could we see production or an importance of IL-18 for NK cells in the Huh6 subgenomic replicon system (*120*) (Fig. 6.6). Zhang et al. traced the NK cell activation back to IFN-α produced by pDCs, although they also saw a role for monocytes, as NK cells + pDCs in co-culture with Huh7.5-JFH1 cells did not activate NK cells, but using NK cells + pDCs + monocytes resulted in NK cell activation. Similar to our observations, NK cells + monocytes alone also did not result in NK cell activation (*120*) (data not shown).

Besides IL-12, IL-18 and IFN-α, other cytokines such as IL-15, IL-10 or IL-23 were already reported in human and mouse studies to facilitate monocyte/macrophage cross-talk (*60*).

Besides cytokine stimulation, cell-cell-contact between monocytes/macrophages and NK cells is often necessary for NK cell activation.

Many studies have demonstrated the expression of NK cell ligands on monocytes and macrophages after stimulation and their interaction with activating receptors, such as NKG2D, 2B4, NKp30, NKp46, NKp80 or DNAM-1 on NK cells, leading to IFN-γ production or cytotoxicity (*60*)(*61*). Haller et al. could also show that Lactobacillus-treated macrophages co-cultured with NK cells led to upregulation of CD25 on NK cells and was dependent on cell-cell-contact. The nature of the interaction was, however, not further defined (*148*).

We identified OX40L as one potential NK-cell activating ligand that is upregulated on monocytes in replicon-containing co-cultures. Monocytes were previously shown to express OX40L after different inflammatory stimuli, including HCV. Piconese et al. could stain OX40L on monocytes, especially on M2-like monocytes, from liver samples of chronic HCV patients (*168*). Furthermore Zhang et al. could show that OX40L expression on peripheral monocytes was induced in chronic HCV patients and that OX40L expression correlated with rapid and early virological response after peg-IFN-α/Ribavirin treatment. As a mechanisms of OX40L upregulation they identified TLR-2-mediated recognition of the viral Core protein (*169*). This cannot be the mechanisms of OX40L induction in our study, because the subgenomic replicon does not contain Core protein. Also, when we, in collaboration with Robert Thimme and Maike Hofmann from the University Hospital Freiburg, addressed OX40L expression on monocytes in a cohort of chronic HCV patients, we could not stain OX40L on peripheral monocytes (data not shown). Both of the above mentioned studies did not address interaction of OX40L-expressing monocytes with NK cells. Piconese et al. focussed on the interaction with Treg cells, which highly express OX40 in non-cirrhotic and tumour liver tissues of HCV patients. Triggering of OX40 on Tregs supports the suppressive phenotype of Treg cells and thereby favours tumour progression. On the other hand, blocking of OX40L on monocytes suppressed the functions of $CD4^+$ T cells and OX40L expression on monocytes in HCV patients was associated with a higher antigen-specific $CD4^+$ T cell response (*169*).

So far, the impact of OX40L expression on monocytes for NK cells was, to our knowledge, never studied. Two studies in mice reported the interaction of OX40L-expressing pDCs and mast cells, respectively, with NK cells and one human study in 2012 by Martinet et al. showed the impact of OX40L on pDCs for NK cell activation in chronic HBV patients (*34*) (*35*) (*37*). Liu et al. show in a B16 tumour model with injection of CpG-activated pDCs that the recruitment to the tumour and activation of T cells is dependent on NK cells. The NK cells themselves are recruited by the activated pDCs via CCR5 and are activated via IFN-α and OX40L, wherein IFN-α primarily stimulates cytotoxicity and OX40L NK IFN-γ production. They could show the upregulation of OX40L on CpG activated pDCs and also show OX40 expression on NK cells. This is, to our knowledge, the only report showing OX40 expression on NK cells (*34*). Vosskuhl et al. also show that IFN-γ production, but not cytotoxicity, of NK cells is partly induced by OX40L expression on LPS-activated Mast cells in mice. They, however, failed to detect corresponding OX40 expression on NK cells (*35*). Both studies indicate that OX40L-OX40 interaction increases IFN-γ production, but not NK cytotoxicity. Although we so far just directly addressed the impact of OX40L on CD25 expression and proliferation (Fig. 6.11) and only showed impaired IFN-γ production after CD14 depletion (Fig. 6.8), the above mentioned findings favour the speculation that OX40L blocking would also decrease IFN-γ production in our system. Moreover, in the human system an effect of stimulated pDCs on IFN-γ production was also observed. CpG-stimulated pDCs from chronic aviremic HBV patients in co-culture with heterologous NK cells produced higher amounts of IFN-γ than NK from co-cultures with unstimulated pDCs. This was not observed with pDCs from viremic patients due to an impairment of pDC activation by the virus. The same was true for NK cell degranulation. Stimulation of NK cells with soluble OX40L and IFN-α enhanced NK degranulation. Of note, IFN-γ production was not addressed, neither was the effect of soluble OX40L alone. Therefore, one could speculate that the effects on NK cell degranulation are primarily mediated via IFN-α in this setting, which would be in line with previous reports. They could furthermore show that OX40L expression on pDCs negatively correlated with viral load and Hepatitis B surface antigen and that the virus interfered with pDC expression of OX40L, but not with other TNFSF members, such as 4-1BBL, ICOSL or GITRL. Although they claim that OX40L interacts with OX40 on NK cells, they do not show OX40 expression on NK cells (*37*).

7.3.1 OX40L expression

Even though we observed upregulation of OX40 on NK cells and of OX40L on monocytes in co-cultures with Huh6-sgJFH1 cells and reduced NK proliferation and CD25 upregulation after blocking of OX40L, we also detected OX40L on Huh6 cells and hepatocytes in patient liver samples (Fig. 6.9, 6.10, 6.11 and 6.12). Because OX40 is expressed at higher levels and on more NK cells only on the replicon-containing conditions, blocking of the OX40L/OX40 axis could primarily affect NK cell activation here, irrespective of the expression of OX40L on monocytes. To distinguish the impact of OX40L on monocytes and hepatocytes, we tried to knock-down OX40L expression in Huh6 cells by shRNA, but so far, this approach was not successful (data not shown). Because Huh7 cells do not express OX40L (data not shown), but show similar NK activation after replicon co-culture (Huh7 9-13), further analysis using OX40L blocking in this setting could also provide answers to this question. Of note, the Huh6 system could be better suited to serve as a model for this pathway *in vitro* because primary hepatocytes in HCV livers, like the Huh6 cells express OX40L, while Huh7 cells do not.

Although we so far cannot tell whether OX40 is activated by OX40L on monocytes or OX40L on the Huh6 cells and we do not know whether in the Huh7 9-13 system NK activation will be dependent on similar pathways, we know that monocytes are important for the virus-dependent activation and that depletion of monocytes and blocking of OX40L had very similar effects on NK CD25 expression. This favours a scenario in which the recognition of OX40L on monocytes is important, but CD14 cells could also provide additional stimuli for NK cells, e.g. to promote upregulation of OX40 or CD25. A possible candidate could be the before mentioned IL-27 (chapter 7.2.1). Although the Transwell experiments pointed to a cell-cell-contact dependent stimulus, cytokines could additionally play a role.

7.4 NK cell activation by different viruses and viral strains

So far, all our findings are based on the Huh6 replicon system using the JFH1 HCV strain, which is genotype 2a. Therefore we further wanted to investigate other cell lines, HCV genotypes and related viruses for their potential to activate NK cells in our co-culture settings. As already discussed in chapter 7.1, we could not see NK cell activation in co-cultures with Huh7-sgJFH1 or HepaRG-sgJFH1 cells (data not shown). However, when the HCV subgenomic replicon 9-13 (genotype 1b) in Huh7 cells was used

in PBMC co-cultures, we did observe NK cell activation (Fig. 6.13), showing that also other HCV genotypes are capable of inducing a similar NK cell activation (significantly higher CD25 levels and IFN-γ production and increased proliferation). Furthermore, it shows that also Huh7 cells can activate NK cells in this setting, depending on the replicon they carry. The viral RNA levels between Huh7-sgJFH1 and Huh7 9-13 cells are comparable. In some experiments 9-13 RNA was slightly higher, in others slightly lower than the JFH1 RNA, which most likely depends on the cell cycle and cell passage (data not shown), excluding the RNA levels as a possible reason for the different effects on NK cell activation. Because NK cells are activated via $CD14^+$ monocytes and they are most likely stimulated via the recognition of viral RNA in exosomes, it is tempting to speculate that the RNA levels present in exosomes is different in the two strains. However, further experiments are needed to determine whether the mechanisms of NK cell activation are the same in the Huh6-sgJFH1 and the Huh7 9-13 system. Establishment of Huh6 9-13 cell lines would also help to differentiate between the influences of the cell lines and the replicons.

When comparing the data from the Huh6 and the Huh7 replicon systems another observation can be made. The "background activation" that is independent of the virus, is much higher in co-cultures with the parental Huh6 or Huh6 cure cell line, than with the Huh7 cell line. The Huh6 cell themselves seem to have a higher potential to activate NK cell than the Huh7 cells. Huh6 and Huh7 cells both express many, but not the same, NK cell ligands, which could be the reason for these differences. One ligand that is differentially expressed by the two cell lines that could be of particular interest is, as already mentioned, OX40L, which is expressed by the Huh6, but not by the Huh7 cells.

To evaluate if the activation of NK cells is specific to HCV or if other viruses can do the same, we co-cultured PBMCs with Huh6 cells containing a subgenomic Dengue virus replicon, another member of the flaviviridae family. NK cells from the Huh6-DENV co-cultures did not show significant differences in the percentage of CD25, proliferation or IFN-γ production when compared to parental Huh6 cells (Fig. 6.13). One reason why Dengue virus did not activate NK cells in our setting, could be that DENV is not known to pack its RNA into exosomes (*170*). This could prevent recognition by monocytes and subsequent NK cell activation. So far the only flaviviridae member that has been shown to do so is HCV. Interestingly, Hepatitis A virus has been shown to be transmitted via viral RNA in exosomes as well and could therefore be an interesting candidate to study (*170*). Furthermore it would be interesting to study DENV in a

system containing free virions, which can be recognised by monocytes to see whether NK cells can be activated in this setting and whether it uses the same pathway.

7.5 Final conclusions

We have described a novel pathway of NK cell activation in Hepatitis C Virus infection, which is dependent on OX40L, $CD14^+$ monocytes and IL-2 to stimulate NK cell proliferation and IFN-γ production.

We could show upregulation of CD25 and OX40 on NK cells after PBMC - Huh6-sgJFH1 co-culture and enhanced proliferation and IFN-γ production of NK cells. This is in line with other reports from T cells and NK cells in mice and humans in which CD25 and OX40 stimulation led to enhanced proliferation, IFN-γ production, but not cytotoxicity. To our knowledge, this is the first report showing the expression of OX40 on human NK cells.

There is accumulating evidence that the interaction of accessory cells and NK cells in HCV is the main route for NK cell activation. Monocytes have been shown to stimulate NK cells in HCV infection via soluble (e.g. IL-18) or cell-cell-contact dependent factors (*122*) (*120*). We identified OX40L as one potential cell-cell-contact dependent factor. So far, the interaction of OX40L on monocytes or hepatocytes and OX40 on NK cells has not been studied and presents interesting opportunities for future investigations.

Besides OX40L and monocytes, IL-2 was also necessary to activate NK cells in our system and this was most likely produced by T cells. However, IL-2 levels were similar in all conditions including PBMCs cultured alone. Therefore production of IL-2 is not virus-dependent and also does not results from allogeneic stimulation of T cells by the hepatocytes.

So far, our experiments were performed with peripheral NK cells and PBMCs from healthy donors and hepatoma cell lines carrying the subgenomic HCV replicon. There is some evidence that the pathway we identified might be of importance for patients and outcome of infection. OX40L expression has been shown in HCV patients on peripheral blood monocytes (*169*), which contributed to an early response to IFN-α/Ribavirin treatment, probably via $CD4^+$ T cells. Vica versa, in HBV patients low levels of OX40L on pDCs was correlated with high viral load and impaired NK cell activation (*37*).

We could show OX40L expression on hepatocytes and other intra-hepatic cells in HCV patient livers (Fig. 6.12). To address whether those include macrophages/ Kupffer cells we want to perform co-staining of OX40L with macrophage/ Kupffer cell markers and furthermore OX40 and NK cell co-stainings. However, further studies are needed to investigate the relevance for the OX40-OX40L pathway for NK cell activation in the liver *in vivo*.

Exploitation of this pathway could be a possible avenue for new HCV treatments. OX40 agonistic antibodies are at present in clinical trials for cancer patients but could also be of use in HCV therapy. In fact, OX40 agonist therapy could have several advantages compared to IFN-α/Ribavirin treatment. In contrast to the type I IFN receptors which are constitutively expressed on a wide variety of hematopoietic and non-hematopoietic cells, OX40 is only expressed on NK cells and on antigen-specific T cells after activation. OX40 expression patterns in the liver have yet to be evaluated, but if OX40 is expressed in areas of viral replication, activation of immune cells could happen locally at the infection sites. This would decrease side effects, which are severe in IFN-α/Ribavirin therapy and often lead to premature termination of treatment. Side-effects in clinical studies using α-OX40 therapy in cancer patients were already reported to be acceptable (*36*).

Furthermore, in contrast to IFN-α, which was shown in many studies to mainly increase NK cell cytotoxicity, triggering of OX40 stimulates IFN-γ production by NK cells and T cells, which has been shown to be more important to virus control than cytotoxicity (*122*) and could also limit the extent of liver injury caused by cytotoxic T and NK cells.

However, treatment with agonistic OX40 antibodies should also be considered with caution, because in mice it was shown that in chronic infections treatment with α-OX40 antibodies can leads to severe $CD8^+$ T cell-mediated immunopathology (*171*).

8 Acronyms

ADCC	antibody-dependent cellular cytotoxicity
ALR	Aim2-like receptor
ALT	alanine aminotransferase
APC	antigen-presenting cell
BCR	B cell receptor
CFSE	carboxyfluorescein succinimidyl ester
DAA	direct acting antiviral
DC	dendritic cell
DENV	Dengue virus
GAS	gamma Interferon activation site
GM-CSF	granulocyte macrophage colony-stimulating factor
HA	haemagglutinin
HCMV	human cytomegalovirus
HCV	Hepatitis C virus
HCV$_{cc}$	cell-culture derived HCV
HCV$_{pp}$	HCV pseudoparticles
HIV	human immunodeficiency virus
HN	Hemagglutinin neuraminidases
HSPG	heparan sulfate proteoglycans
IDU	intravenous drug user

IL-10 Interleukin-10

IL-18BP IL-18 binding protein

ILC innate lymphoid cell

IFN Interferon

IFN-γ Interferon-gamma

iNOS inducible nitric oxide synthase

IRES internal ribosomal entry site

ISG IFN-stimulated genes

ITAM immunoreceptor tyrosine-based activation motif

ITIM Immunoreceptor tyrosine-based inhibitory motif

JAK Janus kinase

JFH1 japanese fulminant hepatitis 1

KIR killer-cell immunoglobulin-like receptor

LCMV lymphocytic choriomeningitis virus

LDL low-density lipoproteins

LDLR low-density lipoprotein receptor

LGP2 laboratory of genetics and physiology 2

LPS lipopolysaccharide

MACS magnetic-activated cell sorting

MAIT mucosal-associated invariant T cell

MAVS mitochondrial antiviral signaling protein

MCMV murine cytomegalovirus

MDA5 melanoma differentiation gene 5

MDSC myeloid-derived suppressor cell

MHC major histocompatibility complex

MIC-A	MHC class I polypeptide-related sequence A
MIC-B	MHC class I polypeptide-related sequence B
MLV	murine leukemia virus
NCAM	neural cell adhesion molecule
NCR	natural cytotoxicity receptor
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cell
NK	Natural killer
NKG2D	Natural Killer group 2, member D
NLR	NOD-like receptor
NO	nitric oxide
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCNA	proliferating cell nuclear antigen
PHH	primary human hepatocytes
PI3K	phosphatidylinositol-3 kinase
PRR	pattern recognition receptor
RAG	recombination-activating gene
RIG-I	retinoic acid-inducible gene-I
RLR	Rig-like receptor
ROS	reactive oxygen species
SCID	severe combined immune deficiency
SNP	single nucleotide polymorphism
SR-BI	scavenger receptor BI

STAT	signal transducers and activators of transcription
SVR	sustained virological response
TAP	transporter associated with antigen-processing
TCR	T cell receptor
TGF-β	transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
TNFR	tumor necrosis factor receptor
TNFRSF	tumor necrosis factor receptor superfamily
TNFSF	tumor necrosis factor superfamily
TRAIL	TNF-related apoptosis inducing ligand
TRAIL-R	TRAIL-receptor
TRAF	TNFR-associated factor
Treg	regulatory T cell
ULBP	UL16 binding protein
UTR	untranslated region
VLDL	very low-density lipoproteins

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