Characterization of NK cell immunity in chronic HCV infection

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**Posterbeiträge**


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Abstract

Natural killer (NK) cell responses are crucial for anti-viral and anti-tumor immunity. However, the immune responses of NK cells are impaired in chronically HCV-infected patients, which can contribute to HCV-related diseases, including hepatocellular carcinoma (HCC). Since 2014, direct-acting antivirals (DAAs) such as Harvoni®, were approved for therapy of chronic HCV patients. While DAA application allowed a complete viral clearance in HCV patients, the recovery of NK cell immunity in these patients is only fragmentary explored and underlying molecular mechanisms are fundamentally uncharacterized.

The first part of this thesis aimed to characterize the molecular phenotype of NK cells in chronic HCV patients before and after DAA treatment. Primary NK cells from HCV patients that were treated with Harvoni® were analyzed before and one year after the start of therapy by using quantitative proteomics, and compared to NK cells from healthy individuals. In total, more than 4867 proteins were identified and quantified by mass spectrometry, covering both the surface markers and intracellular components. Principal component analysis (PCA) and heatmap of correlation factors revealed that NK cell proteomes from patients before treatment were highly different from healthy donors and had a high inter-individual variance. NK cell proteomes of the same patients one year after treatment did normalize their inter-individual variance to the level of healthy individuals but, notably, were found still different from the healthy controls. Among the 308 proteins dysregulated in patients before treatment, only 15% were rescued in the cured patients to the level of healthy controls, including the interferon-stimulated genes (e.g. MX2). The other 85% were sustained dysregulated in W56 NK cells, including the activating receptors (e.g. CD59, and SIGLEC7), inhibitory receptors (e.g. CD300A, GPR56, and LAIR), adaptor proteins (e.g. ADAP, STAM1 and STAM2) and antiviral proteins (e.g. PRMT5 and HMGN2).

In conclusion, these data complement our knowledge of impaired NK cell responses in chronically HCV infected patients and, importantly, indicated both trained and impaired NK cell functions in cured patients.

Among protein regulations that indicated trained NK cell immunity, the adhesion and degranulation-promoting adaptor protein (ADAP) was selected for functional studies. The importance of ADAP and related protein complexes is well characterized in adaptive immunity but its specific role in NK cell immunity is still elusive. Thus, the second part of this thesis
focused on the importance of ADAP in human NK cells and was studied by generating and characterizing ADAP knock-out as well as GFP-ADAP re-expressing NK92 cells. Total proteome and in particular immuno-precipitation coupled mass spectrometry assay identified for the first time the Src kinases-associated phosphoprotein 1 and 2 (SKAP1, SKAP2) as interaction partners of ADAP in human NK cells. In vitro experiments then showed that the absence of ADAP in NK92 cells did not alter proliferation, conjugation formation with target cells or translocation of the microtubule-organizing center towards the immunological synapse. One the other hand, ADAP was found essential for IFN-γ response as well as for the degranulation of lytic granules upon the stimulation of NK cells by K562 tumor cells. Moreover, ADAP was also found to regulate migration and adhesion upon stimulation of CXCR4. Interestingly, ADAP is also required for cytotoxicity of human NK cells through controlling the number of serially killed target cells for each NK cell and the ratio of cytotoxic NK cells. To sum up, these data show that ADAP promotes cytokine production and serial killing through regulating adhesion and migration in human NK cells. These functional studies of ADAP confirmed that the up-regulation of ADAP is a part of trained immunity in cured HCV patients.
Abstrakt

Natürliche Killerzellen (NK) sind für die Immunität gegen Viren und Tumore von entscheidender Bedeutung. Die Immunantworten von NK-Zellen sind jedoch bei chronisch HCV-infizierten Patienten beeinträchtigt, was zu HCV-bedingten Erkrankungen, einschließlich Hepatozellulärem Karzinom (HCC), führen kann. Seit 2014 sind direkt wirkende Virostatika (DAAs) wie Harvoni® für die Therapie chronischer HCV-Patienten zugelassen. Während die DAA-Anwendung eine vollständige Virus-Clearance bei HCV-Patienten ermöglichte, ist die Wiederherstellung der NK-Zell-Immunität bei diesen Patienten nur fragmentarisch erforscht und die zugrundeliegenden molekularen Mechanismen sind grundlegend uncharakterisiert.


Unter den Proteinbestimmungen, die auf eine trainierte NK-Zellimmunität hinwiesen, wurde das die Adhäsion und Degranulation fördernde Adapterprotein (ADAP) für funktionelle Studien ausgewählt. Die Bedeutung von ADAP und verwandten Proteinkomplexen ist für die adaptive Immunität gut charakterisiert, aber ihre spezifische Rolle für die Immunität von NK-Zellen ist noch nicht klar. Daher konzentrierte sich der zweite Teil dieser Arbeit auf die Bedeutung von ADAP in humanen NK-Zellen.
Abstrakt

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ACTBL2</td>
<td>beta-actin-like protein 2</td>
</tr>
<tr>
<td>ADAM</td>
<td>a disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ADAP</td>
<td>adhesion and degranulation-promoting adaptor protein</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>Bcl10</td>
<td>B-cell CLL-lymoma 10</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>BL</td>
<td>before treatment</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BST2</td>
<td>bone marrow stromal antigen 2</td>
</tr>
<tr>
<td>BTN3A1</td>
<td>butyrophilin subfamily 3 member A1</td>
</tr>
<tr>
<td>CARMA1</td>
<td>caspase recruitment domain-containing protein 11, CARD11</td>
</tr>
<tr>
<td>CHS</td>
<td>contact hypersensitivity</td>
</tr>
<tr>
<td>CLDN1</td>
<td>claudin 1</td>
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<tr>
<td>co-IP</td>
<td>co-immunoprecipitation</td>
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<tr>
<td>cSMAC</td>
<td>central supramolecular activation cluster</td>
</tr>
<tr>
<td>DAAs</td>
<td>direct-acting antiviral agents</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DEV</td>
<td>donor-to-donor expression</td>
</tr>
<tr>
<td>DNAM-1</td>
<td>DNAX accessory molecule-1</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF2a</td>
<td>eukaryotic translation initiation factor</td>
</tr>
<tr>
<td>EVH1</td>
<td>ENA/vasodilator-stimulated phosphoprotein homolog 1</td>
</tr>
<tr>
<td>FA</td>
<td>formic acid</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>Gads</td>
<td>Grb2-related adaptor downstream of Shc</td>
</tr>
<tr>
<td>GNLY</td>
<td>granulysin</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>GOBP</td>
<td>gene ontology biological process</td>
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<tr>
<td>GPR56</td>
<td>G-protein coupled receptor 56</td>
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<tr>
<td>H</td>
<td>healthy donors</td>
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<tr>
<td>HAVCR2</td>
<td>hepatitis A virus cellular receptor 2</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCD</td>
<td>higher-energy collisional dissociation</td>
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<tr>
<td>HCMV</td>
<td>human cytomegalovirus</td>
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<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HSCs</td>
<td>hepatic stellate cells</td>
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<tr>
<td>HSPG</td>
<td>heparan sulfate proteoglycan</td>
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Abbreviations

IF         immune fluorescence
IFITM1     interferon-induced transmembrane protein 1
IFN-γ      interferon gamma
IP-MS      immune-precipitation coupled mass spectrometry
IRES       internal ribosome entry site
IRF-3      IFN- regulatory factor 3
IS         immunological synapse
ISGs       interferon-stimulated genes
ITAM       immune-receptor tyrosine-based activation motif
ITGA4      Integrin alpha-4
ITGAL      integrin alpha-L
ITGAM      Integrin alpha-M
ITGAX      Integrin alpha-X
ITGβ2      integrin beta
ITGβ7      integrin beta-7
ITIM       immune-receptor tyrosine-based inhibition motif
iTRAQ      isobaric tags for relative and absolute quantitation
ITSM       tyrosine-based switch motif
IkBα       inhibitor of κB-α
KIR        killer cell immunoglobulin-like receptors
LAIR1      leukocyte-associated immunoglobulin-like receptor 1
LAT        linker of activation of T cells
LC-MS/MS   liquid chromatography with tandem mass spectrometry
LDLR       low-density lipoprotein receptor
LFQ        label-free quantification
LVPs       lipoviroparticles
Mac-1      macrophage antigen - 1
MAD5       melanoma differentiation antigen 5
MALT1      mucosa-associated lymphoid tissue lymphoma translocation gene 1
MAVS       mitochondrial antiviral signaling protein
MCMV       murine cytomegalovirus
MFI        mean fluorescence intensity
MHC-I       major histocompatibility complex class I
MMTS       methylmethanethiosulfonate
MS4A1       SEMA4A and B-lymphocyte antigen CD20
Mst-1       Mammalian STE20-like protein kinase 1
MTOC       microtubule organizing center
NCAM       neural cell adhesion molecule, CD56
NK         natural killer cell
NKT        natural killer T cell
NK-κB       nuclear factor kappa-light-chain-enhancer of activated B cells
NS2        nonstructural proteins 2
OCLN       occludin
PBMCs      peripheral blood mononuclear cells
PBS        phosphate buffered saline
PCA        principal component analysis
PFA        paraformaldehyde
X
Abbreviations

PI3K  phosphatidylinositol-3-OH kinase
PKCθ  protein kinase C theta
PKR   RNA-dependent protein kinase R
PLC   phospholipase C
pSMAC peripheral supramolecular activation cluster
PTB   phosphotyrosine-binding domain
PTPRC protein-tyrosine-phosphatase
RAPL  regulator for cell adhesion and polarization enriched in lymphoid tissues
RIAM  Rap1-GTP-interacting adaptor molecule
RIG-I  RNA helicases retinoic acid-inducible gene I
SDI   Simpson diversity index
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SELPLG HLA class II histocompatibility antigen gamma chain
SH2   Src-homolog 2
SH3   Src-homolog 3
SIGLEC7 sialic acid-binding Ig-like lectin 7
SKAP1 Src kinase-associated phosphoprotein of 55 kDa, SKAP55
SKAP2 SKAP55 homolog
SLMAF7 SLAM family member 7
SLP76 SH2-domain-containing leukocyte protein of 76 kD
SR-B1 scavenger receptor class B type 1
SRP9  signal recognition particle 9 kDa protein
STAMs signal-transducing adaptor molecules
STAT  signal transducer and activator of transcription
SVR  sustained virological response
TBK1  Serine/threonine-protein kinase TBK1
TCEP  tris(2-carboxyethyl)phosphine
TCR   T cell receptor
TEAB  tetraethylammonium bromide
TFA   trifluoroacetic acid
TGF-β1 transforming growth factor β1
TLRs  Toll-like receptors
TRAF6 TNF receptor-associated factor 6
TRAIL TNF-related apoptosis inducing ligand
TRAT1 T cell receptor-associated transmembrane adaptor 1
TRIF  TIR domain-containing adaptor molecule 1
TUBB8 tubulin beta-8 chain
TWF1  twinfilin1
W56   56 weeks after the start of treatment
WT    wild type
YINM  tyrosine-based signaling motif
ZAP70 zeta-chain-associated protein kinase 70
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1. Introduction

1.1. Hepatitis C viral infection

Hepatitis C virus (HCV) is a member of the Flaviviridae family which is characterized by an enveloped, positive-sense RNA. As a blood-borne virus, HCV is mainly transmitted through infected blood and body fluids. The diagnosis for HCV usually includes testing for HCV antibodies in the serum, measuring HCV RNA in the serum, determining viral genotypes and subtypes, and assessing resistance-associated substitutions. Around 30% of the infected persons get only an acute infection, meaning they can clear HCV without any treatment within 6 months, while the remaining 70% of the patients will develop a chronic infection since they cannot rely on their own immune system to clear HCV. More than one hundred million people around the world are estimated to have a chronic HCV infection, and 15% to 30% of these chronic HCV infected patients will develop liver failure, liver cirrhosis and hepatocellular carcinoma (HCC).

1.1.1. Molecular Virology of HCV

HCV is a single-stranded RNA virus with a genome of about 9.5-kb that encodes a single polyprotein (Figure 1). The polyprotein is processed by viral and cellular proteases into three structural proteins (core protein, glycoproteins E1 and E2) and seven nonstructural proteins (i.e., viroporin p7, nonstructural proteins 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B). These nonstructural proteins are of importance for HCV replication. p7 and NS2 are critical for HCV assembly and release after the replication, NS3 functions as RNA helicase and serine protease, NS4B mediates virus-host interaction, NS5A is involved in RNA replication and viral assembly, and NS5B is an enzyme for transcription and replication.

![The HCV polyprotein organization and processing](image)

**Figure 1 | The HCV polyprotein organization and processing.** The HCV RNA genome is translated into a single polyprotein that is further processed into 10 viral proteins. The C-terminal of the core protein is cleaved by signal
peptide peptidase and then packed as a capsid of HCV. The E1, E2 and p7 proteins are generated through cleavage by cellular signal peptidase. E1 and E2 will be packed as envelope glycoproteins and p7 is a viroporin and assembly factor. NS2 and NS3 make up the protease that mediates autocleavage, generating NS2 protein, an assembly factor. NS3 and NS4A then make up the protease and cleave the rest of proteins, including NS3, NS4A, NS4B, NS5A, and NS5B. (from Scheel et al. 2013 6).

1.1.2. HCV life cycle

A complete HCV replication cycle (Figure 2) includes entry into host cells, uncoating of the genome, protein translation, genome replication, and virion assembly and release 7.

**Figure 2 | HCV life cycle.** The viral particle of HCV is first captured by LDLR, and then attaches to the cell surface via interaction with SRB1 and CD81. After attachment, the virus enters the cell by clathrin-mediated endocytosis and releases RNA into the cytoplasm, where HCV RNA is translated into polyprotein and processed to viral proteins. Following RNA replication in the membranous web, all the components are assembled into new viral particles and then released out of the host cell. ER, endoplasmic reticulum; LDLR, low-density lipoprotein receptor; SRB1, scavenger receptor class B member 1. (from Manns et al. 2017 2)

HCV entry is regulated by various factors. Foreign HCV particles are tightly associated with lipoproteins from the host to form lipoviroparticles (LVPs) and transported by blood flow to the surface of the host cell, where they are captured by heparan sulfate proteoglycan (HSPG) and low-density lipoprotein receptor (LDLR). Viral E2 protein of the captured LVPs then interacts with Scavenger receptor class B type 1 (SR-B1), which will lead to E2 conformation change and binding of the viral E1/E2 protein complex with CD81 on the host cell8–10. Next, CD81 with the
bound particle migrates to the tight junction where they interact with claudin 1 (CLDN1) \(^{11}\) and occludin (OCLN) \(^{12}\) that are located there, causing the clustering of CD81 and thereby inducing clathrin-dependent endocytosis of HCV particle. The HCV-containing endosomes are acidified, fuse with lysosomes, and uncoated, ultimately releasing the HCV genome into the host cell cytoplasm \(^{13}\). After release into the cell cytoplasm, the internal ribosome entry site (IRES) in HCV genome enables it to use the host cell translation machinery to synthesize the viral protein \(^7\). Then, the single viral polyprotein is processed by cellular and viral proteases, generating the structural and non-structural proteins as mentioned above (see Chapter 1.1.1.) \(^4\).

The following viral replication takes place at the so-called "membranous web" which provides physical support, protects viral complexes from nucleases and proteinases, and enables efficient replication and structural organization \(^{13,14}\). HCV NS5B, the RNA-dependent RNA polymerase, uses the positive-strand RNA to synthesize the negative-strand RNA. These two RNA strands are then further copied by semiconservative replication to generate multiple positive-strand RNA genomes. Since NS5B lacks proofreading ability, the products of replication have high genetic variability, finally contributing to constant immune escape \(^{15,16}\).

Viral assembly is initiated on the cytosolic side of the ER membrane and is completed in the ER lumen. The NS3 and NS5A proteins are recruited to enable the association of RNA with core protein and the encapsulation of the genome. Then the core protein-containing particles acquire a lipid envelope from the host cell and incorporate the E1 and E2 proteins to form the complete viral particle. Finally, the virus is combined with lipoproteins to generate LVPs and released from the host cell \(^2,16\).

1.1.3. Pathobiology of chronic HCV infection

Due to the absence of suitable small animal models, the process of HCV infection was studied in health-care workers accidentally injured by needle sticks and in experimentally infected chimpanzees \(^{17,18}\). When exposed to HCV, around 30% of patients can spontaneously clear HCV in the phase of acute infections, while the remaining 70% of patients will develop a chronic infection because their own immune systems fail to clear the virus.

As the first line of immune defense, the host innate immune system has the ability to control and potentially clear viral infections. Several HCV RNAs can be recognized by the host cells via pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), RNA helicases retinoic acid-inducible gene I (RIG-I), RNA-dependent protein kinase R (PKR), and melanoma differentiation antigen 5 (MDA5) \(^{19}\). After recognition of viral RNA, these PRRs are activated.
Introduction

and transmit signals to adaptor molecules, like mitochondrial antiviral signaling protein (MAVS) or TIR domain-containing adaptor molecule 1 (TRIF). These adaptor molecules then activate Serine/threonine-protein kinase TBK1 (TBK1), induce translocation of IFN- regulatory factor 3 (IRF-3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NK-κB) into the nucleus, and trigger synthesis of inflammatory cytokines. Through these mechanisms, the virus-infected cells, macrophages and dendritic cells (DCs) produce type I interferons (IFN-α and IFN-β) and type III IFNs (IFN-λ, IL29, IL28A, and IL28B), and natural killer (NK) cells and natural killer T (NKT) cells produce type II IFN (IFN-γ) 17,20,21. These IFNs then bind to their receptors on immune cell surface and activate Janus kinase/signal transducers and activators of transcription (Jak-STAT) signaling to induce expression of interferon-stimulated genes (ISGs). ISGs are the antiviral proteins that inhibit transcription, degrade viral RNA, block protein translation and modify protein function 22. For instance, MX1 expression is associated with spontaneous and treatment-induced HCV clearance 23, MX2 has been reported to interact with NS5A and thereby inhibiting the RNA replication of HCV 24, viperin has been reported to bind NS5A and limit HCV replication 25, and interferon-induced transmembrane protein 1 (IFITM1) interacts with CD81 and OCLN to prevent HCV entry into host cells 26. More ISGs have been recently identified to block HCV replication, although not all mechanisms are fully understood 27–30. Combinations of these ISGs contribute to the control of HCV infection.

However, the expression of these ISGs in most patients fails to clear HCV 31,32 as 70% of HCV-infected persons develop a chronic HCV infection. The mechanisms behind this are still incompletely understood but there are several models presented. The first model is that HCV inhibits the transcriptional level of IFN and ISGs. NS3/4A proteases have been reported to block IFN expression in hepatocytes through cleaving and inactivating MAVS in the RIG-I pathway 33 and TRIF in the TLR3 pathway 34. HCV can also inhibit ISG expression through interfering with Jak-STAT signal transduction 35,36. In the second model, HCV inhibits ISGs translation directly at the ribosomes via phosphorylating protein kinase PKR and eukaryotic translation initiation factor (eIF2a) 37,38. The third model suggests that HCV replication takes place in the subcellular compartment and thus is not accessible to antiviral ISGs 39.

As a consequence of failing to clear HCV, the infected cells in the liver either are damaged or chronically produce inflammatory cytokines. One of the most important cytokines is transforming growth factor β1 (TGF-β1), which can induce quiescent hepatic stellate cells (HSCs) to develop into myofibroblasts 40. The activated HSCs then produce large amounts of abnormal extracellular matrix (ECM), changing the composition of the liver and giving rise to liver fibrosis. Advanced liver fibrosis results in cirrhosis, liver failure and HCC 41,42.
1.2. Natural killer (NK) cell immunity

NK cells are cytotoxic lymphocytes that play a major role in killing virus-infected cells and tumor cells. They originate from CD34+ precursors in the bone marrow and mature in peripheral organs, including blood, liver, skin, gut, spleen, and lung. NK cells comprise about 5-15% of blood lymphocytes and 30-40% of liver-resident lymphocytes and are characterized by the absence of CD3 and expression of CD56 (neural cell adhesion molecule, NCAM). Based on the expression level of CD56, NK cells can be further divided into distinct subsets, named CD56bright and CD56dim cells. In human peripheral blood mononuclear cells, approximately 10% of the NK cells are CD56bright cells and the other 90% are CD56dim cells. It was believed that CD56bright cells have a higher capacity for cytokine production and lower cytotoxic capacity while CD56dim cells produce fewer cytokines but are highly cytotoxic. However, some studies indicate that CD56dim cells can also produce large amounts of cytokines under the stimulation of tumor cells but not by soluble factors. More recently, another new subset of NK cells that do not express CD56 has been identified. These CD56neg NK cells expand in chronic viral infections and show a similar phenotype as CD56dim NK cells but their specific function is still under debate.

1.2.1. Activating and inhibitory receptors

NK cells are regulated by various activating and inhibitory receptors, as well as cytokine receptors. This is in contrast with T cells, whose activation is dominant by the T cell receptor (TCR). The germline-encoded activating and inhibitory receptors on the NK cell surface recognize ligands expressed on target cells. For instance, the NKG2D receptor on NK cells recognizes major histocompatibility complex class I (MHC-I)-related molecules and also UL16-binding proteins. Of these natural cytotoxicity triggering receptors (NCRs), NKp44 and NKp46 on NK cells recognize viral hemagglutinin, and NKp30 binds to BAT3 and B7-H3 on the stressed and transformed cells. The low-affinity IgG receptor CD16 enables NK cells to recognize target cells opsonized with antibodies and kill them in antibody-dependent cell-mediated cytotoxicity (ADCC). 2B4 on NK cells binds to CD48 expressed in the hematopoietic cells. LFA-1 integrin can also contribute to NK cell activation and cytotoxicity through mediating firm adhesion to the target cells. Killer cell immunoglobulin-like receptors (KIRs) recognize MHC class I molecules expressed on healthy cells and NKG2A/CD94 heterodimers can bind to the ligand HLA-E. Furthermore, NK cells can also express receptors for cytokines. Recognition of type I interferon, IL-2, IL-12, IL-15, and IL-18 activates NK cells through the JAK-STAT pathway. IL-12 and IL-18 synergistically stimulate IFN-γ production.
IL-2 promotes NK cell proliferation and activation, and IL-15 promotes NK cell proliferation and maturation.

These activating and inhibitory receptors convey their signals via the motifs that can vary for individual receptors. The activating receptors, NKp30, NKp44, NKp46, and CD16 are coupled with partner chains, which all contain immune-receptor tyrosine-based activation motifs (ITAM), e.g. CD3ζ, FcεRIγ, and DAP12. Another activating receptor, NKG2D, is associated with DAP10 that has a tyrosine-based signaling motif (YINM). The receptors that carry an immune-receptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail, for instance, 2B4, CRACC and NTBA, can either activate or inhibit NK cell immunity. The inhibitory receptors, including KIRs and NKG2A/CD94, are coupled with immune-receptor tyrosine-based inhibition motif (ITIM).

**Table 1** Overview of NK cell activating and inhibitory receptor signaling, ligands and function. The activating receptors associated with adaptor proteins that have an immunoreceptor tyrosine-based activation motif (ITAM) are shown in red. The receptors carried non-ITAM motifs, e.g. tyrosine-based signaling motif (YINM) and immune-receptor tyrosine-based switch motif (ITSM) are shown in yellow. Integrins in blue also contribute to NK cell activation. Inhibitory receptors associated with immune-receptor tyrosine-based inhibition motifs (ITIMs) are shown in green. (Adapted from Bryceson et al. 2011)
1.2.2. NK cells recognition of healthy cells and target cells

All these activating and inhibitory receptors interact with their ligands at the immunological synapse (IS), which is the contact site for NK cells and target cells. Based on the balance of activating and inhibitory ligands on the target cell, NK cell function can be inhibited or activated (Figure 3). Higher expression of inhibitory than activating ligands on healthy cells induces tolerance of NK cells. Either losing of MHC-I expression or overexpression of stress-induced ligands on tumor cells can activate NK cells, resulting in the release of cytokines and cytotoxic molecules through the activating synapse (see also legend Figure 3).

**Figure 3 | Recognition of healthy cells and target cells by NK cells.** (A) Healthy host cells express more inhibitory ligands (such as MHC-I), inducing more inhibitory than activating signals inside NK cells, resulting in the tolerance of NK cells. (B) Some tumor cells lose the expression of MHC-I to avoid T cell immunity. The missing of MHC molecules leads to a weaker inhibitory than activating signal, promoting activation of NK cell and lysis of tumor cell. (C) Other tumor cells up-regulate stress-induced ligands, which then stimulate more activating receptors on NK cells and promote the killing of tumor cells (from Vivier et al. 2012 56)
The activation of NK cells begins with the formation of peripheral supramolecular activation cluster (pSMAC), the peripheral region of the IS, where the integrins LFA-1 and Mac-1 mediate the adhesion and conjugation between the NK and the target cell (Figure 4A). Activation of LFA-1 initiates actin polymerization, causing accumulation of filamentous actin at the pSMAC, which paves the way for polarization and transport of lytic granules towards the microtubule organizing center (MTOC). Concomitantly, other activating receptors accumulate in the central supramolecular activation cluster (cSMAC), the central area of the IS, and promote the cytotoxic signaling. The synergistic activating signaling in NK cells is similar to the activating signaling pathways in T and B cells. In brief, ligation of receptors promotes phosphorylation of ITAM, ITSM or YISM by Src family tyrosine kinases, followed by the recruitment of zeta-chain-associated protein kinase 70 (ZAP70) and Syk. Then ZAP70 and Syk phosphorylate transmembrane protein linker of activation of T cells (LAT) and cytosolic adaptor protein SH2-domain-containing leukocyte protein of 76 kD (SLP76). These recruited adaptor molecules then further activate phosphatidylinositol-3-OH kinase (PI3K), phospholipase C (PLC-γ1/2) and Vav (Vav1/2/3), which facilitate regulation of actin cytoskeleton, calcium ion (Ca^{2+}) flux and granule exocytosis (Figure 4B).

1.2.3. Adaptor molecules in NK cell signaling networks

NK cells tend to express several receptors that are redundant for one specific function. The redundant signals from activating and inhibitory receptors have to be coordinated and integrated
by multiple adaptor molecules, for example, the DAP12, DAP10 and protein-tyrosine phosphatase SHP-1 and SHP-2. DAP12 has an ITAM in its cytoplasmic domain and therefore can associate with several receptors, e.g. NKP44, KIRs, CD94/NKG2C. Ligation of these receptors with their ligands induces Src-family kinase-mediated tyrosine phosphorylation of the ITAM motif in DAP12. Then DAP12 recruits and phosphorylates tyrosine kinases Syk and ZAP70, which further phosphorylate transmembrane adapter molecules LAT and NTAL. The phosphorylated LAT and NTAL leads to activation of several signaling complexes, e.g. phosphatidyl-inositol-3-OH kinase (PI3K), phospholipase C (PLC-γ1, PLC-γ2) and Vav1/2/3. PI3K regulates the calcium flux and Vav-1/2/3 initiates actin reorganization and polarization of DAP10 contains a YINM motif and associates with NKG2D. When NKG2D is activated by its ligands, the YINM motif in DAP10 is phosphorylated by Src-family kinases and directly activates the signaling complexes, including PI3K, Grb2, PLC-γ1, PLC-γ2 and Vav1. SHP-1 and SHP-2 are associated with the inhibitory receptors, e.g. inhibitory KIRs, NKG2A, and LAIR. Ligation of these inhibitory receptors with the corresponding ligands leads to the phosphorylation of their ITIM motif by Src-family tyrosine kinase. Facilitated by β-arrestin 2, the phosphorylated ITIM recruit and phosphatase SHP-1 and SHP-2, which result in the de-phosphorylation of Vav1 and thus inhibition of actin cytoskeleton rearrangement and polarization of MTOC. Furthermore, there are also adaptor proteins that not only transduce signals but also function as a scaffold for protein interaction, e.g. adhesion and degranulation-promoting adaptor protein (ADAP).

1.2.4. Adhesion and degranulation-promoting adaptor protein (ADAP)

As a multifunctional scaffolding protein, ADAP has no enzymatic activity but can facilitate the formation of signalosomes. ADAP was first identified as an interaction partner of the tyrosine kinase Fyn and the adaptor protein SLP-76. In human, the ADAP gene is found on chromosome 5 (5p13.1, Ensembl ENSG00000082074) and the ADAP protein contains 783 amino acids. Until recently, ADAP has been mostly studied in T cells but has also been reported in other immune cells, including NK cells.

1.2.4.1. Structure and binding partners of ADAP in T cells

ADAP contains a proline-rich sequence (PRS), two helical SH3 domains (hSH3), several tyrosine-based signaling motifs and an Ena-Vasp homolog binding domain. As it shows in Figure 5, the proline-rich sequence in the N-terminus and the SH3 domain of the C-terminus mediate the constitutive binding of ADAP to Src kinase-associated phosphoprotein of 55 kDa (SKAP55, SKAP1) and the SKAP55 homolog (SKAP-HOM, SKAP2). The two hSH3 domains
mediate transient interactions with lipid membranes. As indicated by the name, the EVH1 domain interacts with the ENA/vasodilator-stimulated phosphoprotein homolog 1 (EVH1). The tyrosine-based signaling motifs facilitate binding to SH2 domains of other signaling proteins, for example, Y^{595} and Y^{651} enable binding to SLP-76, Y^{625} mediates interaction with Fyn kinase and Y^{595} is also responsible for binding with tyrosine kinase adaptor protein NCK1.

In addition to these structural features, a lot of other interaction partners (Figure 5) have been characterized using in vitro kinase assays with mutation of tyrosine to phenylalanine. These potential binding abilities enable ADAP to function as a scaffold protein, regulating the downstream signal transductions.

**Figure 5 | Tyrosine-phosphorylation (pY)-dependent interaction partners of ADAP in T cells.** Schematic representation of ADAP primary structure including unstructured regions (light blue) and the two structured hSH3 domains (green). FYN-dependent tyrosine-phosphorylation sites identified by LC-MS/MS are highlighted in red. Phosphorylation-dependent SH2 domain-containing interaction partners were identified from pulldown experiments using in vitro phosphorylated protein constructs (top) and short synthetic peptide motifs (bottom) are indicated by black arrows. Phosphorylation-independent interactions are indicated by grey arrows (review in and taken from Kuropka, B. et al. 2016).

### 1.2.4.2. ADAP regulates integrin activation and NK-κB signaling in T cells

Most of our knowledge about ADAP was achieved in T cells, where ADAP couples TCR stimulation or chemokine receptor stimulation with integrin-mediated T cell activation, termed as “inside-out signaling” (Figure 6, upper part). The most important binding partners of ADAP in this process are SKAP1 and SKAP2. Overall, around 70% of ADAP molecules in T cells are constitutively bound to SKAP1 and all SKAP1 and SKAP2 molecules are bound to ADAP to
stabilize its expression. Following TCR activation the ADAP/SKAP1 module is recruited to a complex consist of linker for activation of T cells (LAT), Grb2-related adaptor downstream of Shc (Gads) and SLP-76. Afterwards, the ADAP/SKAP1 module either binds to Rap1-GTP-interacting adaptor molecule (RIAM) and Mammalian STE20-like protein kinase 1 (Mst-1) to form ADAP/SKAP1/Mst1/RIAM/Kindlin-3 complex or binds to regulator for cell adhesion and polarization enriched in lymphoid tissues (RAPL) and kindling-3 to form ADAP/SKAP1/RAPL/Mst1 complex. Both of these two complexes play a role in activating integrin LFA-1. In the case of chemokine receptors (CCR7 or CXCR4) mediated LFA-1 activation, these two complexes, ADAP/SKAP1/Mst1/RIAM/Kindlin-3 and ADAP/SKAP1/RAPL/RAPL/Mst1, are also needed.

**Figure 6 | Role ADAP in T cell activation.** In T cells 70% of ADAP is associated with SKAP55 (SKAP1) for the regulation of integrin signaling. When TCR or chemokine receptors are stimulated, ADAP/SKAP1 complex interacts with RIAM/Mst1/Kindlin-3/Rap1 and the RAPL/Mst1/RAPL complex to promote the inside-out signaling for LFA-1 activation as well as the out-side in signaling for T cell adhesion and migration, or interaction with target cells. The other 30% of ADAP that is not bound to SKAP1 regulate cell cycle and NF-κB signaling upon TCR/CD28 activation. Through interaction with TAK1/PKCθ, ADAP promotes CDK2 and cyclin E expression for cell cycle regulation. Via interaction with PKCθ/CBM/TRAF6, ADAP facilitates NF-κB entering the nucleus and initiating gene transcription. (adapted from Witte A. et al. 2012).

The other 30% of ADAP proteins are required for TCR/CD28-mediated activation of NF-κB, necessary for controlled activation and proliferation of T cells.
resting T cells, NF-κB is inactivated due to binding of the inhibitor of κB-α (IκBα) in the cytoplasm. Upon TCR activation, protein kinase C theta (PKCθ) phosphorylates caspase recruitment domain-containing protein 11 (CARMA1, CARD11) which then forms the CBM complex with B-cell CLL-lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1). The CBM complex interacts with E3 ubiquitin protein ligase TNF receptor-associated factor 6 (TRAF6) to facilitate polyubiquitination and degradation of IKKγ. Meanwhile, PKCθ also activates TAK1 to phosphorylate IKKβ. IKKγ degradation and IKKβ phosphorylation result in IKKα release, IκBα phosphorylation, translocation of NK-κB into the nucleus, and finally the initiation of target genes transcription.

By binding the CBM complex, ADAP facilitates activation of TRAF6 and IKKγ degradation, and through direct interaction with TAK1, ADAP regulates IKKβ phosphorylation. Therefore, in both processes, ADAP promotes IKKα release and NK-κB translocation. In conclusion, in T cells, ADAP forms a signalosome with PKC0/CBM/TRA6 or TAK1 that facilitate NF-κB activation and transcription of targeted genes.

1.2.4.3. ADAP function in NK cells

Until now, the ADAP function in NK cells is still incompletely understood and investigated. Killing (See chapter 1.3) capacity of murine ADAP−/− NK cells did not show a significant difference to wild type NK cells when stimulated with YAC-1, CHO, C1498.D4, EL4 and P815 cells.

Neither 2B4 antibody nor IL-12 stimulated IFN-γ production by murine NK cells was affected by ADAP knockout. Likewise, LFA-1-mediated conjugation in NK cells did not require ADAP. However, through interacting with SLP76, ADAP could regulate degranulation and IFN-γ production by Ly49D stimulation. Interestingly, upon NKG2D and CD137 stimulation, ADAP was reported to be crucial for mediating full cytokine responses, but dispensable for murine NK cells cytotoxicity. Similarly, knock-down of ADAP in human primary NK cells affects exclusively cytokine production but not cytotoxicity. Recently, Böning et al. reported that ADAP is required for degranulation and migration of murine NK cells during the in vivo infection of Listeria monocytogenes. Therefore, it is now tempting to speculate that ADAP selectively regulates major NK cell responses in mice, but the role of ADAP in human NK cell immunity is still elusive.

1.3. NK cell effector function

NK cell effector functions against target cells are usually achieved in three ways: (i) secreting cytokines and chemokines; (ii) releasing lytic granules to the IS; (iii) induction of apoptosis through death receptors.
The stimulation of cytokine receptors (e.g. IL-12 and IL-18) or activating receptors (e.g. NKG2D, NKp30) on NK cells induces the production of pro-inflammatory cytokines and chemokines, including IFN-γ and TNF-α, CCL3, CCL4 and CCL5. These secreted cytokines and chemokines, in turn, recruit and stimulate additional NK cells and other immune cells, facilitating clearance of target cells.

Following cell contact-dependent signal transduction in activated NK cells, the lytic granules rapidly move along microtubules in a dynein-dependent manner and converge in the proximity of the MTOC (Figure 4B). For degranulation, the MTOC and the lytic granules polarize to the IS, where the centrosomes can anchor to the cell cortex. The polarization is accompanied by actin cytoskeleton reorganization and reorientation of cell organelles, such as the nucleus, Golgi apparatus, and mitochondria. The polarized mitochondria maintain Ca\(^{2+}\) influx to trigger exocytosis of the vesicles. The polarized lytic granules transit through minimally permissive hypodensities in the F-actin network, dock at the plasma membrane in the IS, and fuse locally with the plasma membrane. After fusing with the plasma membrane, lytic granules release pore-forming proteins perforin and the serine protease granzymes which will diffuse into IS. The fusion of NK cell lytic granules, known as degranulation, has two modes: the complete fusion and incomplete fusion. In complete fusion, the content of the granule is completely diffused into the IS. In incomplete fusion, a transient pore is formed and the granule releases only parts of the content. The rest of the proteins in the granules are recycled into the cytoplasm via Munc13-4 clathrin-mediated endocytosis, probably preparing for the serial killing (see chapter 1.4.). Both fusion and secretion are also regulated by actin, which generates a force to squeeze out the granular contents, tethers granules at the cortex, and acts as a fence to prevent lateral diffusion. The perforins in the IS move toward the target cell to form pores in the membrane so that granzymes can enter the target cell and induce apoptosis. Meanwhile, LAMP-1 (CD107a) accumulates in a cluster at the IS of NK cells, which is used as a marker for degranulation. Recently, CD107a has also been shown to promote the delivery of granzymes B and perforin to the target cell, and protect NK cells from degranulation-associated damage.

Additionally, activating signaling can also induce the expression of pro-apoptotic Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) on the NK cell surface to mediate killing of target cells. FasL is stored in secretory granules that are distinct from classic cytotoxic granules and these FasL containing granules are brought to the NK cell surface by degranulation. After diffusion within the synapse, FasL can bind to its CD95 receptor on target cells and induce CD95 polymerization, which initiates apoptotic signaling in the target cell that involves
the caspase cascade\textsuperscript{89}. Similarly, upon activation, also TRAIL is transported to the NK cell surface where it can interact with the pro-apoptotic receptors, TRAIL-R1 and TRAIL-R2, on the target cell inducing its apoptosis\textsuperscript{90}.

1.4. NK cell serial killing

Following the lysis of target cells the membrane of target cells flips, leading to exposure of phosphatidylserines. These exposed phosphatidylserines may interact with CD300a on the NK cell surface and terminate its response by down-modulation of CD16 and NKG2D\textsuperscript{91–93}. After the detachment from the target cell, NK cell functions can be restored by IL-2 stimulation, which stimulates the generation of new granules and new effector molecules\textsuperscript{82}.

However, some NK cells have the ability to continue killing more target cells without a restoration. Their killing activity is a strictly serial process: kill only one target cell at a time, termed as serial killing\textsuperscript{94,95}. Benefiting from the recycling of the content of lytic granules\textsuperscript{84}, NK cells have the ability to finish up to six rounds of killing despite the down-regulation of activating receptors\textsuperscript{96}. Interestingly, NK cells that perform serial killing have been reported to switch to death receptor-mediated killing when perforin and granzymes are insufficient\textsuperscript{97}.

1.5. NK cell memory—trained immunity

Although NK cells are classically regarded as innate immune cells, some recent evidence suggests that NK cells exhibit adaptive features as well. These features include the expansion of pathogen-specific NK cells and the existence of long-lasting “memory” NK cells, so-called “trained” innate immunity\textsuperscript{98–100}. Three main types of NK cell memory have been described: Hapten-specific memory, murine cytomegalovirus (MCMV)-specific memory, and cytokine-induced memory\textsuperscript{98,101–103}.

Trained immunity of NK cell was first described in mice lacking T cells and B cells that could still develop hapten-specific contact hypersensitivity (CHS) (Figure 7A)\textsuperscript{104}. Here, only a subset of liver-resident NK cells in the mouse developed memory against various haptons and other antigens\textsuperscript{105,106}. To develop a memory for antigens, also cytokine signals are required, including IL-12, IFN-γ, IFN-α and IL-18\textsuperscript{107,108}.

Also in the case of CMV infection, NK cell functions are altered. In C57BL/6 mice NK cells can specifically recognize the MCMV ligand m157 because the corresponding receptor is required for NK cell survival in MCMV infection (Figure 7B)\textsuperscript{109}. In human, a unique subset of NK cells with a mature phenotype (CD56\textsuperscript{dim}CD57\textsuperscript{+}NKG2A\textsuperscript{+}) expand to adapt to human CMV (HCMV) infection\textsuperscript{110–112}. This HCMV-adapted NK cell expansion can be driven by NKG2C or co-
stimulatory receptor CD2 or lacking the intracellular signaling adaptor FceRγ\textsuperscript{113–115}. Epigenetic modifications in the HCMV-adapted NK cells that alter intracellular signaling proteins and transcription factors contribute to the altered functions \textsuperscript{116,117}.

Figure 7 | Pathways for the generation of memory NK cells. (A) Hapten-specific memory NK cells are generated from naïve NK cells following sensitization of hapten and simulation by cytokines, including IL-12, IFN-γ and IFN-α. These memory NK cells can develop contact sensitivity responses after the hapten challenge, which is regulated by NKG2D, CXCR6, CD18, and selectins. (B) MCMV infected cells express m157 and CD155, ligands for LY49H and DNAM-1, which will induce the expansion of naïve NK cells into effector NK cells with the stimulation of IL-12, IL-18, and IL-33. Following the elimination of virus-infected cells, BIM and autophagy mediate the contraction of effector NK cells, leaving only MCMV-specific memory NK cells distributed systematically. (C) Naïve NK cells cultured with IL-12, IL-15 and IL-18 up-regulate the expression of IFN-γ, perforin and granzymes, as well as CD25, the receptor for IL-2. These cytokine-induced memory NK cells will continue to produce a higher amount of IFN-γ, perforin and granzymes after adoptive transfer. (Adapted from Cerwenka et al. 2016 \textsuperscript{101}).

Cytokine stimulation alone can enhance NK cell immunity in the long-term (Figure 7C). In mice, splenic NK cells pre-activated with IL-12/IL-15/IL-18 and transferred to naïve hosts displayed higher IFN-γ and perforin-mediated cytotoxicity when stimulated with activating receptors, cytokines and tumor cells. These pre-activated NK cells are dependent on IL-2 from CD4\textsuperscript{+} T
cells and macrophages for proliferation and long-term function\textsuperscript{118-121}. Similarly, human NK cells \textit{in vitro} stimulated with IL-12/15/18 and maintained with IL-15 had enhanced IFN-γ production when stimulated with cytokines and acute myeloid leukemia blasts\textsuperscript{117}, and higher killing ability against K562 tumor cells\textsuperscript{118}.

\subsection*{1.6. NK cells in HCV infection}

Known as antiviral and antitumor immune effector, NK cells have the ability to kill HCV-infected hepatocytes\textsuperscript{124}. Although HCV mainly infects the liver, most investigations focused on human peripheral blood NK cells instead of liver-resident NK cells, because liver biopsies are usually not available and contain only limited amounts of cells for research\textsuperscript{125}. Despite the differences between NK cells from peripheral blood and liver, evidence shows that the NK cell responses observed in periphery blood are similar\textsuperscript{126} or even stronger\textsuperscript{127} than in the liver.

Emerging substantial reports suggests that NK cells are important for controlling HCV replication during chronic HCV infection and treatment\textsuperscript{125,128,129}. Although the frequency of total NK cells in chronic HCV infection has been reported as either unchanged or reduced, decreases of CD56\textsuperscript{dim} cells ratio in NK cells are frequently reported in the peripheral blood of patients, in comparison with healthy donors\textsuperscript{130-132}. As CD56\textsuperscript{dim} cells produce fewer cytokines but are highly cytotoxic\textsuperscript{133}, the lower ratio of CD56\textsuperscript{dim} cell potentially caused impaired NK cell cytotoxicity.

Still, the findings on NK cell receptor expression in chronic HCV infections are contradictory. For example, the expression of NCRs (NKp30, NKp44, and NKp46) and NKG2D on peripheral NK cells has been reported to be up-, down-regulated or unchanged in independent studies\textsuperscript{126,131,134,135}. However, the heterogeneity of research backgrounds made it difficult to compare and validate these findings\textsuperscript{129}. Likewise, conclusions are also contradicting regarding the cytotoxic function of NK cells in chronic HCV infection, reported as unchanged\textsuperscript{130,131}, enhanced\textsuperscript{126} and impaired\textsuperscript{136}. Lunemann \textit{et al.} reported that NK cells in acute and chronic HCV infection displayed lower degranulation (CD107a) upon IFNα stimulation\textsuperscript{136}. Ahlenstiel \textit{et al.}, however, suggested that NK cells in chronic HCV patients polarize toward their cytotoxic function due to higher expression of TRAIL, NKp44, and NKG2C, which contributes to liver inflammation and injury. In contrast, cytokine production, e.g. IFNγ, is reported in most of the studies as impaired in the peripheral NK cells from chronic HCV patients\textsuperscript{126,131,137,138}. The distinct expression of KIR on NK cells and the corresponding HLA ligands influence the outcome of the acute and chronic HCV infection\textsuperscript{126}. Patients with inhibitory KIR2DL3-expressing NK cells have higher chances to clear acute HCV infection\textsuperscript{139}, whereas patients with activating KIR2DS3-expressing NK cells are less likely to clear HCV\textsuperscript{140}. 
Immobilized and concentrated E2 protein from HCV virions may cross-link CD81 on the NK cell surface and inhibit its function \(141-143\). Direct contact with HCV-infected hepatoma cells have been reported to down-regulate NKG2D and Nkp30 expression on NK cell surfaces and reduce NK cell functional capacities \(144\), which is potentially associated with the NS3 and NS4A protease \(145\). Finally, HCV RNA released from infected hepatoma cells promotes cytokine secretion in accessory cells, such as monocytes \(146\), Kupffer cells \(147\), macrophages and dendritic cells \(148,149\), leading to sustained activation of NK cells.

### 1.7. Therapy for chronic HCV infections

More than 100 million chronic HCV-infected patients are estimated at risk of severe diseases, including liver cirrhosis and hepatocellular carcinoma (HCC). The diagnosis and treatment strategies for HCV infections experienced continuous progress, benefiting from the intimate interplay between basic, translational and clinical research \(2,6\). The treatment of HCV began in 1986, when hepatitis C was still named as non-A, non-B hepatitis, by injecting patients with recombinant IFN-\(\alpha\) three times a week \(150\). Successful treatment is defined as the blood is negative for HCV RNA 24 weeks after therapy, known as the sustained virological response (SVR). Later, the monotherapy with IFN was replaced by the combination of PEGylated interferon plus ribavirin, which has dominated HCV treatment for more than 10 years \(151,152\). However, IFN-\(\alpha\) Ribavirin only applies to certain patients and causes severe side effects.

Treatment with direct-acting antiviral agents (DAAs) has revolutionized the treatment of chronic HCV patients because more than 90% of the patients achieve SVR \(153\). DAAs directly target multiple steps of the HCV life cycle and can be classified into four groups (Figure 8) according to in which step they interfere—namely, NS3/4A protease inhibitors (e.g. Paritaprevir), NS5B nucleoside polymerase inhibitors (e.g. Sofosbuvir), NS5B nonnucleoside polymerase inhibitors (e.g. Dasabuvir), and NS5A inhibitors (e.g. Ledipasvir and Daclatasvir) \(154\).
Introduction

Figure 8 | Direct-acting antiviral agents inhibit HCV replication. Different steps of HCV replication are targeted: (A) NS3/4 protease inhibitors target HCV translation and polyprotein processing; (B) NS5A inhibitors target RNA replication; (C) NS5B inhibitors target RNA replication and virion assembly; (D) cyclophilin inhibitors target transport and release of the HCV virion. (from Poonia et al. 2015 155).

1.8. Effects of DAA treatment on NK cell responses

Despite the successful clearance of HCV by DAA therapy, several reports suggested unexpected increased rates of HCC development and recurrence 156,157, although this was not observed in all studies 158. DAA-mediated viral clearance is accompanied by normalized levels of ISGs in the liver and in blood 159. Yet another study showed that the altered cytokine and chemokine milieu in the blood in chronically infected HCV patients were not completely restored by a successful Sofosbuvir and Ribavirin treatment 160, showing the incomplete knowledge of the mechanisms how DAA treatment affects individual patients.

Immediately following the availability of DAA therapy, NK cell immunity under DAA treatment has been studied 159,161–163. Already in 2015, two publications suggested that NK cell function was normalized by the successful interferon-free DAA therapy, as revealed by decreased expression of activating receptors on NK cells, decreased cytotoxicity and increased cytokine production of NK cells in cured patients 159,163. Jiang et al. suggested DAA treatment downregulated the expression of NKp46 and NKG2A on NK cells but increased their ability to kill HCV-infected cells 161. Li et al. also reported that the expression of NKP30, NKP46, and NKG2A on patient NK cells were normalized to the level of healthy controls162. However, another study reported heterogeneous data, suggesting DAA treatment (Sofosbuvir/Ledipasvir) made no difference in IFN-γ production and NK cytotoxicity against K562 cells, which were transiently activated during therapy and then dampened to the baseline level before treatment 164. Strunz et al. found no significant difference in the expression of 13 surface markers and immune responses in NK cells from HCV patients before and after treatment, compared with healthy
control. Interestingly, Strunz et al. also assessed the diversity of NK cells and found that following DAA treatment the donor-to-donor expression variation (DEV) was normalized while the Simpson diversity index (SDI) stayed reduced (Figure 9). DEV is a novel method for estimating the variation of immune cells between individuals. The increase of DEV is associated with severe liver fibrosis. SDI is a metric adapted from ecology for measuring the diversity of cell populations inside each individual. Reduced SDI implies the expansion of discrete subsets of NK cells due to chronic HCV infection. Therefore, the chronic HCV infection might result in the expanding of various subsets of NK cells, implying the disturbance of NK cell immunity.

Figure 9 | Imprint on NK cell diversity by chronic HCV infection. (A) Based on the markers measured by flow cytometry, the donor-to-donor expression variation (DEV) was calculated to assess the inter-individual variability. (B) The intra-individual diversity of the NK cell population was assessed by the Simpson diversity index (SDI). (C) Longitudinal assessment of chronic HCV infection patients before, during, and after DAA treatment. The elevated DEV in chronic HCV patients was normalized in parallel with DAA treatment and improvement of liver stiffness. In contrast, the reduced NK cell SDI remained lower than in healthy donors. (Strunz B. et al. 2018).
2. Aims of the study

Natural killer (NK) cell responses are crucial for anti-viral and anti-tumor immunity and essentially controlled by germline-encoded activating and inhibitory receptors. NK cell responses are impaired in patients who are chronically infected by the hepatitis C virus (HCV), but the underlying mechanisms hereof are still elusive. Fortunately, the approval of direct-acting antiviral agents (DAAs) since 2014 has enabled the complete clearance of HCV in most of the patients within 8 weeks. Thus, this successful therapy offers us a chance to observe the NK cell response along with and after HCV clearance. Although several studies have reported on the NK cell immunity after DAA therapy, their conclusions are inconsistent and mainly based on only a few surface markers, thus leaving open questions in the field of anti-HCV therapy.

The first part of this thesis aims to systematically evaluate NK cell function in chronic HCV patients before and after DAA therapy, and to answer the following questions:

1) What are the molecular phenotypes of NK cell in chronic HCV infected patients?
2) Is NK cell immunity in the cured patients rescued, still impaired or even trained?

To answer these questions, proteomic analyses have been applied to compare NK cells obtained from chronic HCV infected patients before treatment (BL) and 56 weeks after the start of DAA treatment (W56), as well as healthy donors (H) as control. Bioinformatics tools supporting these clinical proteome approaches have been selected and combined to evaluate the molecular phenotypes of NK cells. The potential role of the significantly regulated proteins and enriched pathways has been assessed in the context of NK cell immunity and HCV infection.

NK cell immunity requires adaptor proteins to integrate signals from various receptors. These adaptors, e.g. adhesion and degranulation-promoting adaptor protein (ADAP), not only function as scaffolds for protein interactions but also transduce signals to downstream responses. However, in contrast with the extensive studies on the role of ADAP in T cells, its specific role in NK cells is still incompletely understood. Only a few publications have inconsistently reported on the role of ADAP in mice NK cells.

Therefore, the aim for the second part of the thesis is to clarify the importance of ADAP in regulating NK cell functions, specifically to answer the following question:

3) What is the role of ADAP in human NK cell immune responses?

This question have been addressed on wild type, ADAP knock-out and rescued NK92 cells by performing in vitro functional assays, including interferon gamma production, degranulation, cytotoxicity, adhesion and migration, and serial killing assays. In addition, immune-precipitation-coupled mass spectrometry (IP-MS) has been performed to identify the interaction partners of ADAP in human NK cells.
Aims of the study
3. Materials and Methods

3.1. Equipment and Software

3.1.1. Equipment

BD Accuri C6 Flow Cytometer, Accuri Cytometers Inc.
BD LSR II SORP Cytometer, BD Biosciences
Innova CO-170 CO₂ incubator, New Brunswick Scientific
Inverted microscope Ti-E, Nikon
Orbitrap Fusion™ Tribrid™, Thermo Scientific
LTQ Orbitrap Velos Pro™ Fourier Transform, Thermo Scientific
LAS-3000 CCD-camera, Fujifilm
RC10101 SpeedVac, Sorvall
Sorvall discovery M120 SE Centrifuge
UltiMate 3000 HPLC, Dionex Centrifuge
Axio Observer 7 microscope in Zeiss ApoTome, Carl Zeiss AG

3.1.2. Software

BD Accuri CFLOW Sampler 1.0.264.21, Accuri Cytometers Inc.
BD Accuri CFLOW Analysis Plus 1.0.264.21, Accuri Cytometers Inc.
BioEdit v7.0.5, Informer Technologies, Inc.
FACSDiva 6.1, BD Biosciences
FlowJo 10.0.7, TreeStar Inc.
Inkscape 0.92.4
MaxQuant 1.6.2.3
Microsoft Excel 2010, Microsoft
NIS-Elements AR 4.30.20, Nikon
ProteomeDiscoverer 2.2, Thermo Scientific
Perseus 1.6.2.3
RStudio 1.1.456.0, RStudio Inc
3.2. Reagents

3.2.1. Buffers, media and antibodies used on cells

If not indicated otherwise, all used chemicals were obtained from the following companies: PAA Laboratories (Pasching, Austria), Life Technologies/Invitrogen/Gibco (Carlsbad, CA, USA), Sigma Aldrich/Fluka (St. Louis, MO, USA), Carl Roth GmbH (Karlsruhe, Germany) and BD Biosciences (Franklin Lakes, NJ, USA).

All buffers and solutions were prepared with MilliQ water, obtained from a MembraPure Astacus purification system (Membrapure GmbH, Bodenheim, Germany). All media and buffers used for cell culture were sterilized by filtering through Steritop Filter Units (Merck Millipore).

3.2.2. Cell culture media:

RPMI complete: RPMI-1640, 10% fetal bovine serum (FBS) gold, 2 mM L-glutamine, 1% Penicillin + Streptomycin
alpha-MEM medium: alpha-MEM, 12.5% FBS gold, 12.5% horse serum (FIRMA), 2 mM L-Glutamine, 1% penicillin + streptomycin
K562 medium: RPMI-1640 medium, 10% FBS gold, 1% penicillin + streptomycin
Migration medium: (RPMI1640 containing 10mM HEPES (pH 7.4)
DMEM medium: DMEM, 10% FBS gold, 1% penicillin + streptomycin
LB medium: 15 g/L yeast extract, 10 g/L tryptone, 7 g/L sodium chloride
LB agar: LB medium with 15 g/L agar

3.2.3. Solutions and buffers

Phosphate buffered saline (PBS): 1.37 mM sodium chloride, 2.7 mM potassium chloride, 12 mM phosphate (HPO$_4^{2-}$/H$_2$PO$_4^{2-}$)
Gel buffer A: 1.44 M Tris Base, 0.4% SDS, pH 8.8
Gel buffer B: 0.48 M Tris Base, 0.4% SDS, pH 6.8
Gel running buffer: 0.24 M Tris Base, 1.99 M glycine, 1% SDS
4 × Sample buffer: 4% SDS, 20% glycerol, 200 mM DTT, 0.01% bromophenol blue and 0.1 M Tris HCl, pH 6.8
Blotting buffer: 25 mM Tris Base, 192 mM glycine, 20% methanol, 0.02% SDS
TBS: 0.2 M Tris Base, 1.37 M NaCl, pH 7.6
TBS-T: TBS + 0.1% Tween 20

FACS buffer: PBS, 2% fetal bovine serum FBS, 1 mM ethylenediaminetetraacetic acid (EDTA)

IF fixation solution: PBS, 4% paraformaldehyde (PFA)

IF permeabilization solution: PBS, 0.15% Triton X-100

IF blocking solution: PBS, 1% bovine serum albumin (BSA), 0.05% Tween-20

IF washing solution: PBS, 0.05% Tween-20

Urea lysis buffer: 1 M tetraethylammonium bromide (TEAB) supplemented with 8 M urea

Lysis buffer: 1% Triton-X-100, 0.5% SDS, 0.5 M TEAB, 1% Complete protease inhibitor (Roche, Cat.04693116001, MilliQ water

Reducing reagent: 50 mM tris(2-carboxyethyl)phosphine (TCEP)

Cysteine blocking reagent: 200 mM methylmethanethiosulfonate (MMTS) in isopropanol

RP Binding buffer: 3% acetonitrile (ACN) in 0.2% trifluoroacetic acid (TFA)

RP Eluting buffer: 60% ACN in 0.2% TFA

BRP buffer A: 1% ACN in 10 mM Ammonium hydroxide

BRP buffer B: 90% ACN in 10 mM Ammonium hydroxide

MS buffer A: 0.1% formic acid (FA)

MS buffer B: 80% ACN in 0.1% FA

3.2.4. Antibodies

All primary and secondary antibodies used are shown in Table 2 (Flow cytometry), Table 3 (primary antibodies for immune fluorescence microscopy, Western-Blot and co-immunoprecipitation (co-IP)) and Table 4 (secondary antibodies for IF and WB)

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<th>Host</th>
<th>Clone</th>
<th>Manufacturer</th>
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Materials and Methods

Table 3 | Primary antibodies used for immune fluorescence microscopy and Western-Blot

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Table 4 | Secondary antibodies used for immune fluorescence microscopy and Western-Blot

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3.3. Label-free quantification of NK cells from HCV patients

3.3.1. PBMCs collection and NK cells isolation

NK cells from 6 chronic HCV infected patients and 6 healthy donors were collected by Dr. Julia Hengst in the department of Gastroenterology, Hepatology and Endocrinology, headed by Prof. Dr. Heiner Wedemeyer at Hannover Medical School. The clinical data of these donors are in Table 5. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors and patients at different time points along with Harvoni treatment and immediately frozen at -80°C. NK cells were sorted by flow cytometry based on the expression of CD3-CD14-CD19- and CD56+ on single cells. Afterwards, these NK cells were washed, frozen as pellets, and shipped to the Helmholtz Centre for Infection research.

Table 5 | Clinical data of HCV patients and healthy donors

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</tbody>
</table>
3.3.2. Cell lysis and protein digestion

NK cell pellets were lysed with 8 M urea in 1 M TEAB (triethylammonium bicarbonate) complemented with Benzonase for 30 min at RT. As a reducing agent, 5 mM tris(2-carboxyethyl) phosphine (TCEP) was added and incubated for 30 min at 25°C. Then the cysteine blocking reagent, 10 mM methylmethanethiosulfonate (MMTS), was added and incubated for 30 min at room temperature in the dark to modify reduced sulfhydryl groups to dithiomethane. For digestion, 1 µg combined LysC/trypsin reagent (Promega) was added in each sample and incubated for 5 h at 25°C, following by dilution of urea by 700 μL MilliQ water from 8 M to 1 M and incubation at 37°C overnight. To check digestion efficiency, 5% of the digested solution was cleaned by Ziptip (See Chapter 3.3.3) and analyzed using Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) with an 80 min gradient (See Chapter 3.3.5.). Via RawMeat, the distribution of peptide charges was visualized and calculated.

3.3.3. Peptide cleaning with Ziptips

To clean minimal amounts of peptides, Ziptips were first activated by pipetting 20 µL RP Eluting buffer (60% ACN / 0.2% TFA), followed by twice washes with 20 µL RP Binding buffer (3% ACN / 0.2% TFA). Then the peptide sample was pipetted twice through the Ziptip. Afterwards, the Ziptip was washed twice with 20 µL RP Binding buffer and eluted with 20 µL RP Eluting Buffer. The eluate was then vacuum-dried in SpeedVac concentrator and resuspended with 10 µL RP Binding buffer. The resuspended sample was ultra-centrifuged at 50,000 × g for 20 min at 20°C and transferred to an HPLC vial for MS measurement.

3.3.4. Peptide cleaning with reverse phase column

The rest of the sample was acidified with 50 µL 10% Formic Acid, spun down at 1000 × g for 10 min, and then cleaned up with Oasis reverse phase columns (Waters Corporation). The column was first activated by 1 mL RP Eluting buffer, then washed twice with 1 mL RP Binding buffer. Then the peptide sample was applied twice onto the column, followed by two times washing with 1 mL RP Binding buffer and eluting by 200 µL BP Eluting buffer. The eluate was then vacuum-dried in the SpeedVac and resuspended with 100 µL RP Binding buffer. The resuspended sample was ultra-centrifuged at 50,000 × g for 20 min at 20°C and transferred to an HPLC vial for MS measurement.
3.3.5. **Peptide sequencing (LC-MS/MS)**

The cleaned peptides were analyzed separately by a Dionex UltiMate 3000 RSLCnano LC system (Thermo Scientific) connected to an LTQ Orbitrap Fusion™ Tribrid™ Fourier transform mass spectrometer (Thermo Scientific). Before the final measurement, a short gradient method was used to analyze every sample to calculate the peptide amount. Peptides were loaded onto a C18 pre-column (3 µm RP18 beads, Acclaim, 75 µm x 20 mm), washed for 3 min at a flow rate of 6 µL/min and separated on a C18 analytical column (3-µm, Acclaim PepMap RSLC, 75 µm x 25 mm, Dionex) at a flow rate of 350 µl/min via a linear 30 min gradient from 97 % MS buffer A to 25 % MS buffer B, followed by a 15 min gradient from 25 % MS buffer B to 62 % MS buffer B. The LC system was operated with the Chromeleon software (version 6.8, Dionex) embedded in the Xcalibur software suite (version 3.0.63, Thermo Scientific, Dreieich, Germany). The effluent was electro-sprayed by a stainless steel emitter (Thermo). Peptide fragmentation was carried out using the higher-energy collisional dissociation (HCD) mode in the ion trap.

3.3.6. **MaxQuant processing of MS data**

The MS/MS raw data files were processed by MaxQuant’s 1.6.2.3 integrated search engine Andromeda searching against a UniProtKB/Swiss-Prot protein database of *Homo Sapiens*. Before starting the search, MMTS as a modification was configured into MaxQuant with the composition of “H(2)CS”, position as “Anywhere”, type as “Standard”, new terminus as “None” and specificities as “C”. The parameters used are as follows: MMTS as fixed modifications, maximum number of modifications per peptide as 3, first search peptide tolerance as 10 ppm and main search peptide tolerance as 5 ppm, maximum miscleavage as 1, and the Label-free quantification (LFQ) was turned on. LFQ minimum ratio count was set to 1 and the rest of the parameters are used as default settings.

3.4. **Statistical analysis of proteomic data**

3.4.1. **Determination of significantly regulated proteins**

The statistical assessment of MaxQuant data was achieved using software Perseus 1.6.2.3 and R. The proteinGroups.txt in the MaxQuant output folder was loaded into Perseus, with the LFQ intensity of 54 measurements as main columns. First, the data were cleaned by filtering out the proteins that labeled as “+” in column “only identified by site”, “Reverse” and “Potential contaminant”. Then the gene ontology (GO) annotation file for Homo sapiens was used to annotation, including “GOBP name”, “GOCC name” and “KEGG name”. Based on the information of each measurement, the 54 main columns were grouped into 18 Samples (codes...
of patients) and 3 Groups (H, BL and W56). Then all the intensity values were transformed to log2 values, where a 0 value would be transformed into missing value “NaN”. Then all the log2 values were filtered based on valid values and the threshold was set as the minimum valid number 15 out of 18 in at least one group. After filtering, the three technical replicates from the same sample were averaged by taking the median value. All the left missing values in each column were replaced by imputed Gaussian distribution values with width as 0.3 fold standard deviation and downshift as 1.8 fold standard deviation. Finally, the Hawaii plot (multiple volcano plots) was created, using the healthy group as the negative control, level A threshold as S0=0.2 and FDR=0.01, class B threshold as S0=0.1 and FDR=0.05. All the proteins determined as significantly regulated in BL or W56 were labeled “+” in the corresponding column.

3.4.2. Correlation analysis, principal component analysis and hierarchical clustering

Based on the averaged and imputed dataset, correlation factors of all the NK cell proteomes were calculated using Pearson correlation. Then principal component analysis (PCA) was performed to visualize the projection of data sets defined by PCA in 1 and 2 dimensional viewers.

3.4.3. Visualization of protein expression

To better visualize the expression of each protein in all the samples, the data used for the Hawaii plot in Perseus was exported to a txt file and then imported into R. The function for generating the plot for protein expression was created based on reshape2, ggplot2 and ggpubr packages in R (code #1 in Supplementary file S1). According to the ID or gene name of a specified protein, the expression information was extracted and merged with the sample name as variable ID and LFQ abundance as a variable value. The group information (H, BL and W56) was then added accordingly. The boxplot plus jitter plot was finally created using the group as the x-axis and LFQ abundances as the y-axis, annotated with the p-value of Student’s t-test.

3.4.4. Pathway enrichment of selected proteins

To determine the global regulation of pathways and functions among the samples, the data used for the Hawaii plot in Perseus with annotation of GO categories were analyzed based on the description by Pan et al. 167 and the R script adapted from Voigt et al. 168. For each comparison of BL/H or W56/H, R code #2 (Supplementary File S2) was used to divide all proteins into five regulation groups according to both level A and level B regulation (See Chapter 3.4.1.), and direction of fold change. The first and fifth regulation groups include level A significantly regulated proteins, the second and fourth group include level B significantly regulated proteins, the third group the not-regulated proteins.
Two-sided Fisher’s Exact test was then performed (R code #3 in Supplementary File S3) for assessing the p-value of all the GO categories, with “TRUE” for over-representation and “FALSE” for under-representation. Functions that were significantly (p<0.05) enriched in at least one of the groups were filtered and used to construct a heatmap. To make the heatmap of GO annotation data, the p-values were transformed into log10 values and adjusted to the direction of regulation: the over-represented GO categories with “TRUE” remained positive while the under-represented GO categories with “FALSE” were adjusted to negative values. Euclidean distance and average linkage were used for hierarchy clustering, where +/-0 indicate the direction of regulation and color shows the p-value.

3.5. Functional investigation of ADAP in human NK cells

3.5.1. Cells and culturing

K562 cells were cultured in RPMI complete medium. Phoenix-AMPHO (ATCC® CRL-3213™) cells were cultured in DMEM medium. NK92 cells were cultured in α-MEM medium supplemented with 100 U/ml IL-2. All cells were cultured in culture flasks (Nunc) at 37°C supplemented with 7.5% CO₂.

3.5.2. CRISPR/Cas9 knock-out ADAP in human NK92 cells

Signal-guide RNAs sgRNA_for and sgRNA_rev (Table 6) targeting human ADAP were designed in the webpage http://crispr.mit.edu/ (Zhang Lab, MIT, 2015), synthesized by Eurofins and then cloned into pSpCas9(BB)-2A-GFP (Addgene plasmid no. 48138) vector through the BbsI restriction site according to instructions. The reconstructed plasmid was then transfected into NK92 cells by electroporation via the Amaxa Nucleofector device and R kit, program A24. After 48 h, GFP positive NK92 single cells were sorted by Dr. Lothar Gröbe using Aria-II SORP flow cytometer to 96-well round bottom plates. The single-cell clones were expanded and Western Blot was used for testing ADAP expression.

To detect the genomic microdeletions, the mutation site of the genome in ADAP/ cells was cloned by PCR using primers ADAPseq_for and ADAPseq_rev. The PCR products were cloned into the TOPO vector (Thermo Fischer) according to the manual and sequenced by Eurofins. The sequencing results were compared with data in wild type genome to determine the insertion of deletion of bases.
Table 6 | Primers used in this thesis

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgRNA_for</td>
<td>5'</td>
<td>CACCCGACGGGGGGCAACCCGACAG</td>
</tr>
<tr>
<td>sgRNA_rev</td>
<td>3'</td>
<td>AAACCTGTGGGTGCCCCCGTGCC</td>
</tr>
<tr>
<td>ADAPseq_for</td>
<td>5'</td>
<td>GAAAGGCCAGATGTAAAGTCCC</td>
</tr>
<tr>
<td>ADAPseq_rev</td>
<td>3'</td>
<td>CACTGTGAAGAGATGGCTTG</td>
</tr>
<tr>
<td>GFP-ADAP_Ex-for</td>
<td>5'</td>
<td>CCGCTAGCGCTACCGGGATCCACCATGCTAGGC</td>
</tr>
<tr>
<td>GFP-ADAP_Ex-rev</td>
<td>3’</td>
<td>CAATGACTAGGGGTCGACGGATCTAGATACTG</td>
</tr>
</tbody>
</table>

3.5.3. Re-expression of ADAP in ADAP<sup>−</sup> NK92 cells

GFP-tagged ADAP sequence was amplified using primers GFP-ADAP_Ex-for and GFP-ADAP_Ex-rev from pEGFP-C3-ADAP plasmid, offered by Stefanie Kliche (OvGU, Magdeburg). Then the sequence was cloned into pBMN-IRES-GFP through restriction sites of BamH1 and Sal1. The constructed plasmid, named as GFP-ADAP-pBMN, was used for producing retroviral particles. Retroviral particles were produced using the Phoenix-ampho retroviral packaging cell line, obtained from Dr. Garry Nolan, created by introducing genes producing gag-pol and env for infection of mammalian cells. 2 × 10<sup>5</sup> Phoenix cells were cultured in 6 mL RPMI complete medium in a 6-well plate until about 80% confluence. The following day, 5 µg GFP-ADAP-pBMN or 5 µg pBMN-IRES-GFP was mixed and incubated with 5 µL Lipofectamine P3000<sup>TM</sup> in 125 µL OPTI-MEM medium for 15 min at room temperature. Then 7.5 µL Lipofectamine<sup>TM</sup> 3000 reagent in 125 µL OPTI-MEM medium was added to the plasmid and incubated for another 15 min at RT. The mixture of plasmid and Lipofectamine reagent, together with 750 µL OPTI-MEM medium, were added on top of Phoenix cells and incubated for 3 h at 37°C. Following the addition of another 3 mL RPMI complete medium, the cells were cultured in the incubator for generating retroviral particles.

The supernatant of virus particles was collected and filtered through a 0.45 µm membrane to remove the remaining Phoenix cells. For each transfection, 1 mL virus supernatant, mixed with 5 µL P3000<sup>TM</sup>, 7.5 µL Lipofectamine<sup>TM</sup> 3000 reagent and 6 µM BX795, was used to resuspend 2 × 10<sup>5</sup> NK92 cells. The cells were then transferred to a single well in a 12-well plate, centrifuged for 1 h at 700 × g at RT and then incubated for 3 h at 37°C. The centrifugation and incubation were repeated once again and 2 mL IL-2 containing α-MEM medium was used to resuspend the cells for overnight culture. The GFP positive NK92 cells were then sorted with help from Dr. Lothar Gröbe and expanded for western blot and other functional assays.
3.5.4. Gel electrophoresis and western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the standard protocol in our lab. Buffer A (Tris Base 181.7 g/L, SDS 4 g/L, pH 8.8), MilliQ water and Acrylamide were used for the separation gel, Buffer B (Tris Base 60.6 g/L, SDS 4 g/L, pH 6.8), MilliQ water and Acrylamide were used for the stacking gel. Samples were treated with 1 × Sample buffer supplemented with 1 µL Benzonase, shaking at 25°C for 20 min at 800 rpm and then heated at 95°C for 10 min. The treated samples were then separated on the gel with running buffer (0.24 M Tris base, 1.99 M glycine and 1% SDS) at first for 120 V for 15 min and then 160 V for 50 min. Prestained protein ladders were used for the estimation of molecular weight.

After separating by SDS-PAGE, the proteins were transferred from gel to methanol activated PVDF membrane in blotting buffer (25 mM Tris base, 192 mM glycine, 20% methanol and 0.02% SDS) for 1 h at 60 mA. The membrane was then blocked for 1 h at RT in blocking buffer (5% skimmed milk and 0.1% tween-20 in TBS) and incubated with the first antibody diluted in blocking buffer overnight at 4°C. Following 10 min sequential wash at RT with TBST (0.1% tween-20 in TBS), TBST + 0.5 M NaCl and TBST + 0.5% Triton X-100, the membrane was incubated with the second antibody diluted in blocking buffer for 1 h at RT. Afterwards, the membrane was washed again three times with TBST buffer (10 min) and once with TBS buffer (10 min). Finally, the membrane was illuminated with ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Chalfont St. Giles, UK) using the LAS-3000 CCD-camera (Fujifilm, Tokyo, Japan).

3.5.5. Proliferation assay

For proliferation assays, 2 million WT or ADAP−/− NK92 cells were stained with 7 µM CFSE in 0.1% FBS/PBS buffer for 20 min in the dark at room temperature. Afterwards, the cells were washed twice with α-MEM medium to stop the staining process and cultured at the concentration of 3 × 10^5/mL in a flask with α-MEM medium supplemented with 1000 U/mL IL-2. For measurement, 100 µL NK92 cells were taken out every 24 h, washed with FACS buffer and the CFSE signal was analyzed with Accuri® 6. The median fluorescence intensity of CFSE was used as an indicator of proliferation.

3.5.6. Conjugate assay

According to the products manual, K562 cells were stained with PKH67 (SIGMA) and WT or ADAP−/− NK92 cells were stained with PKH26 (SIGMA) for 5 min at room temperature. 1 mL
FBS was added to stop staining and then the cells were washed twice and cultured in RPMI medium for 2 h to minimize dye transfer later on. The stained NK92 cells were incubated with stained K562 cells in U-bottom 96-well plates for 60, 50, 40, 30, 20, 10, 5 and 0 min, fixed by ice-cold 4% PFA and analyzed by Accuri® 6. The percentage of conjugated NK92 cells equals the sum of PKH26 and PKH76 double positive cells divided by the number of PKH26 positive cells.

3.5.7. MTOC translocation assay

K562 cells were labeled with CFSE as described in NK cell killing assay. NK92 cells and CFSE-labeled K562 cells both resuspended with RPMI complete medium at a concentration of 2 × 10^6 cells/mL were mixed at effector to target (E:T) ratio of 1:1. Then 30 µL of the mixture was applied on poly-L-lysine coated coverslips and settled down for 30 min at 37°C in an incubator supplemented with 7.5% CO₂. Afterwards, the cells on the coverslips were washed once with 30 µL PBS followed by 20 min fixation at -20°C with 30 µL 1 mM EGTA in methanol. The fixed cells were washed once with 30 µL PBS, permeabilized for 5 min at RT with 30 µL IF permeabilization solution (0.15% triton-100 in PBS) and blocked for 1 h with 30 µL IF blocking solution (1% BSA, 0.5% Tween20 in PBS). The first antibody YL-1/2 (Rat anti-human tubulin) diluted 1:2 in 30 µL IF blocking solution was added on top of the cells and incubated overnight at 4°C. After three times washing with IF washing solution (0.05% Tween20 in PBS), the secondary antibody C13c diluted 1:250 in 30 µL IF blocking solution was added on coverslips and incubated for 1 hour at RT. The coverslips were washed twice with 0.5 mL IF washing solution and once with0.5 mL PBS and then dehydrated by 70% ethanol and 100% ethanol. Subsequently, the coverslips were air dried and mounted on slides using heated Mowiol. The next day microscopy was performed on an inverted microscope (Ti-E; Nikon), using NIS-Elements AR software (Nikon).

3.5.8. Adhesion and migration assay

96-well plates were pre-coated with Fc-tagged human ICAM-1 (10 µg/ml; R&D systems) or Fibronectin (5 µg/ml; Roche) in PBS containing 1 µg/ml BSA. After washing with PBS, wells were incubated in the absence or presence of CXCL12 (1 µg/ml) for 60 min at 37°C. NK-92 cells were left untreated or stimulated with MnCl₂ (1 mM) for 30 min at 37°C. Subsequently, cells were allowed to adhere for 30 min at 37°C. Unbound cells were carefully washed off with Hanks balanced salt solution (HBSS; Biochrom AG). Bound cells were counted and calculated as % input (2 × 10^5 cells) in triplicates.
Materials and Methods

Migration assays were performed using Transwells (5.0 μm pore size; Costar) coated with Fc-tagged human ICAM-1 (10 μg/ml; R&D systems) or Fibronectin (5 μg/ml; Roche) for 1h at 37°C and washed two times with PBS (Biochrom AG). NK-92 cells were washed two times in RPMI1640 (Biochrom AG) and resuspended in migration assay medium (RPMI1640 containing 10 mM HEPES (pH 7.4) and 0.1% w/v BSA (Fraction V; PAA)). 0.5 × 10⁶ cells per well were incubated for 2h at 37°C in the presence or absence of human CXCL12 (200 ng/ml; lower chamber; Biolegend). Upon addition of 0.1M EDTA (pH7.3) to each well of the lower chamber for 10 min at room temperature, the number of migrated cells to the lower chamber was counted and calculated as % input. Adhesion and migration assays were performed by Dr. Stefanie Kliche (OvGU, Magdeburg).

3.5.9. Degranulation and IFN-γ production assay

The protocol used for degranulation and cytokine production was adapted from a previous report. NK92 cells were resuspended with α-MEM medium supplemented with 100 U/mL IL-2, distributed into round-bottom 96-well plates without or with the indicated combined stimulation of IL-12 (10 ng/mL), IL-18 (100 ng/mL), K562 (E: T = 1:1), PMA (20 ng/mL) and Ionomycin (500 ng/mL). The cells were spun down at 120 × g for 3 min to increase cell contact. The cells were incubated at 37°C for 1 h before Monensin and Brefeldin A were added into each well to stop the transportation of proteins in NK92 cells. The plate was then spun down again at 120 × g for 3 min and incubated at 37°C for another 5 h. After washing once with PBS, all wells were resuspended with 80 μL PBS supplemented with 20 μL Fc blocking reagent and 0.1 μL Zombie live/dead staining solution, incubated at 4°C for 15 min. For extracellular staining, the cells were stained with CD56-BV786 and CD107a-PerCP-cy5.5 antibodies for 30 min at 4°C. For intracellular cytokine staining, the cells were permeabilized with Cytofix/Cytoperm (BD) solution and washed with Perm/Wash buffer (BD). Then IFN-γ-BV520 and CCL3-FTTC antibodies were incubated for 30 min and washed with Perm/Wash buffer again before acquired with LSR-II SORP (BD Biosciences, v6.1) and analyzed using FlowJo (Tree Star Inc.) and R. Each condition was performed as triplicate. The Mean Fluorescence Intensity (MFI) and percentage of cytokines were normalized by the corresponding non-stimulated value. Student’s t-test was used to determine significant differences.

3.5.10. Cytotoxicity assay

For the killing assay including GFP or GFP-ADAP overexpressed cells, NK92 cells were stained with CFSE using the same protocol as in proliferation assay (Chapter 3.5.5). The stained NK92 cells and K562 cells were resuspended with RPMI complete medium without phenol red.
supplemented with 100 U/mL IL-2, mixed at 4:1 of E: T ratio in 96-well plates with round bottom. The plates were centrifuged at 120 × g for 3 min to increase cell contact and then incubated at 37°C for 4 h. After incubation 7-AAD Live/Dead staining solution was added to the cells at 1:100 and incubated for 5 min then analyzed by Accuri® 6 flow cytometer.

\% of K562 apoptosis = (7-AAD+ K562 cells / K562 cells).

\% of specific lysis = apoptosis with the existence of NK92 - apoptosis without NK92.

3.5.11. Serial Killing assay

For imaging of serial killing events of NK92 cells, a silicon-glass microchip containing one hundred 350 μm-wide wells was used. After seeding K562 target cells, NK92 effector cells (labeled with CellTracker Deep Red, Thermo Fisher) were added in medium containing 100 U/ml IL-2 (final concentration) and 1 μM dead cell dye SYTOX Blue (final concentration). This resulted in a stochastic distribution of approximately 5-12 NK92 cells and 60-100 target cells per microwell. Cells were visualized via time-lapse microscopy using a Zeiss ApoTome System with an Axio Observer 7 microscope equipped with a 20×/0.8 Plan-Apochromat objective and an incubation chamber with environmental control (37 °C, 5% CO₂, humidity device PM S1). Fluorescence of SYTOX Blue was acquired in line sequential mode. SYTOX Blue was excited using the Colibri 7 LED-module 475 (filter set 38 HE LED) and CellTracker Deep Red 555 (filter set 64 HE LED), and a bright-field image was acquired using the TL LED module. Images were acquired every 3 min for 16 h using an AxioCam 506 mono camera. Serial killing assays were performed by Dr. Isabel Prager in the Department of Immunology headed by Prof. Dr. Carsten Watzl in Leibniz Research Centre for Working Environment and Human Factors (IfADo, Dortmund).

3.6. Proteomic analysis of NK92 cells

3.6.1. Proteomic analysis of NK92 cells

The indicated NK92 cells were lysed with 100 µL lysis buffer (1% Triton X-100, 0.5% SDS, 1 × Roche protease inhibitor, 0.5 M TEAB, and 1 Unit Benzonase, filled up with MilliQ water) for 20 min at 22°C. Then the solution was 5 mM TCEP was added and incubated for 30 min at 22°C, following by adding 10 mM MMTS and incubated for 30 min at 22°C in the dark. Subsequently, 10 µL of 30 µg/µL carboxylate beads and 300 µL ethanol were added and shaken overnight at 22°C for 3 h at the speed of 1200 rpm. Then the beads were centrifuged down for 5 min at 5,000 rpm and the supernatant was discarded. Next, the beads were washed once with 80%
ethanol and three times with acetonitrile, then air-dried at RT for 10 min. Then 20 µL iTRAQ dissolution buffer and 13 µL MilliQ water were used to resuspend the beads, 2 µL trypsin were added for overnight digestion at 37°C. The eluted peptides were then labeled with iTRAQ (See Chapter 3.6.3.) and analyzed by LC-MS/MS (See Chapter 3.6.4.).

3.6.2. Immune precipitation coupled with mass spectrometry

For IP experiments, 10 million of WT or ADAP+/− NK92 cells expressing either GFP or ADAP-GFP were resuspended with 5 mL RPMI medium and incubated at 37°C for 15 min, with or without stimulation of 10 million K562 cells. Then the cells were washed twice with 10 mL PBS, resuspended with 5 mL of 0.5% formaldehyde, and incubated at room temperature for 10 min. Afterwards, the crosslinking was quenched by resuspending with 10 mL ice cold 0.25 M glycine. After another two times wash with ice cold PBS, the cells were lysed in 200 µL lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 1 × Roche protease inhibitor, 1000 U/mL Benzonase) with extensive pipetting and sonication. The lysate was centrifuged at 20,000 × g for 10 min at 4°C and the supernatant was transferred to a pre-cooled Lowbind vial. Then equilibrated GFP-Trap beads in 800 µL wash buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) was added and mixed constantly for 2 hours at 4°C. After incubation, the beads were spun down at 2,500 × g for 2 min at 4°C and washed three times with 500 µL ice cold wash buffer. The beads were eluted twice with 100 µL elution buffer (200 mM glycine pH 2.5) and heated at 95°C for 10 min before used for western blot or mass spectrometry analysis. The eluate was neutralized with 20 µL 1 M Tris base (pH 10.4), mixed with 600 µL acetonitrile and shaken with 40 µg carboxylate beads at 22°C overnight at 12,000 × rpm. After centrifugation at 5,000 × g for 5 min, the beads were washed with 200 µL 70% ethanol and then with 200 µL acetonitrile. Then the air-dried beads were resuspended with 20 µL TEAB, mixed with 2 µL 50 mM TCEP for 30 min and then 1 µL 200 mM MMTS for another 30 min. The samples were digested with 1 µg trypsin overnight at 37°C. Afterwards, 500 µL acetonitrile were added and shaken at 25°C overnight. Then the beads were centrifuged down at 5,000 × g for 5 min, washed with 200 µL acetonitrile and eluted with 20 µL 1% DMSO. The eluted peptides were then labeled with iTRAQ (see chapter 3.6.3.) and analyzed by LC-MS/MS (see chapter 3.3.5.).

3.6.3. iTRAQ labeling

The supernatant-containing digested sample was then transferred to a new vial and 34 µL MilliQ water was used to wash the beads and then combined with the previous digestion solution. After checking digestion efficiency with 5% of the sample, labeling of tryptic peptides with isobaric
tags for relative and absolute quantitation (iTRAQ) reagents was performed according to manufacturer’s guidelines (Applied Biosystems, Waltham, MA, USA). Briefly, iTRAQ reagents and 70 µL Ethanol were added to the samples and incubated for 2 h. The samples were dried in a speedvac and resuspended with RP Binding buffer (0.2% TFA/3% ACN), cleaned up by Oasis reverse phase columns (Waters Corporation, Milford, MA, USA) and combined at the same amount of peptides.

3.6.4. Reverse Phase Fractionation and peptide sequencing (LC-MS/MS)

The combined iTRAQ-labeled samples were then resuspended in BRP Buffer A (1% ACN/10 mM NH₄OH) and fractionated on a ZORBAX 300Extend-C18 column (Agilent) connected to an ÄKTA purifier (GE Healthcare) system. The flow started at 1 mL/min for 60 min with a linear gradient from 0% to 80% of BRP Buffer B (90% ACN/10 mM NH₄OH), followed by another linear gradient from 80% to 100% of buffer B for 4 min. All the elutions were collected and combined into 15 fractions. As described above, the peptides were vacuum-dried and resuspended in 100 µL of RP Binding buffer and analyzed separately by a Dionex UltiMate 3000 RSLCnano LC system (Thermo Scientific) connected to an LTQ Orbitrap Fusion Tribrid Fourier transform mass spectrometer (Thermo Scientific). The MS/MS raw data files were processed by Proteome Discoverer searches against the UniProtKB/Swiss-Prot protein database of Homo sapiens.
4. Results

4.1. Characterizing effects of chronic HCV infection on NK cell immunity

4.1.1. Establishment of the workflow for proteomic analysis

This thesis aims to clarify the molecular phenotype of NK cells in chronic HCV patients before and after DAA treatment. In collaboration with Dr. Julia Hengst in the department of Gastroenterology, Hepatology and Endocrinology at the Medical School of Hannover (MHH), blood samples from Harvoni®-treated HCV patients were collected before, along with and one year after therapy (Figure 10A). These patients were all infected with HCV genotype 1b and their age ranged from 56 to 59 at the beginning of the treatment. HCV viral loads in peripheral blood and liver function were checked at baseline (BL=start of therapy), and 1 week (W1), 4 weeks (W4), 8 weeks (W8), 12 weeks (W12), 20 weeks (W20) and 56 weeks (W56) after the beginning of the therapy. In the following chapters of this thesis, “BL” refers to chronically HCV-infected patients and “W56” refers to Harvoni®-treated, HCV-free patients at week 56 after the start of an 8 weeks DAA treatment.

At the end of treatment, viral RNA levels of HCV in the patients were reduced to undetectable, and none of the patients had HCV relapses at one year after the start of the therapy (Figure 11A). Following the clearance of HCV, liver inflammation, as indicated by the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the blood, was decreased (Figure 11 B&C). Peripheral blood mononuclear cells (PBMCs) s from patients at all the time points and healthy donors were isolated and stored at -80°C for further analyses. NK cells from six patients were sorted, using flow cytometry by gating on the CD3−CD4−CD14−CD19−CD56+ cell cluster, thus including both CD56bright and CD56dim NK cell subsets (Supplementary Figure S1). The absolute numbers of lymphocytes were relatively stable (Figure 11D). Since previous reports have suggested that the percentage of CD56dim NK cells in total NK cells is down-regulated in patients with chronic HCV infection 130-132, NK cell numbers and the frequency of major NK cell subsets were evaluated. Neither significant difference in absolute NK cell numbers nor in the ratios of CD56dim NK cells in total NK cells were detected (Figure11 E&F).

To investigate the molecular phenotype of NK cells in chronically HCV patients before (BL) and after treatment (W56), I used label-free quantitative proteomics for analyzing blood samples from six HCV patients at BL and W56, and compared the data with six healthy control samples, with triplicates for each sample (BL: 6 × 3, W56: 6 × 3, healthy: 6 × 3) (Figure 10 B). The MS
Results

spectra were processed with MaxQuant software for the identification and quantification of the proteins. Then, the proteomic data were first evaluated in Perseus, where samples from BL and W56 of HCV patients, and healthy donors were grouped accordingly (H, BL, and W56). Plots were generated using R software to visualize the regulation information for each identified protein across all the samples, as exemplified by MX2 expression across all the samples (Figure 10 B).

**Figure 10 | Workflow for clinical patient analyses and proteomics phenotyping of NK cell.** (A) Blood samples from six Harvoni®-treated HCV patients before (baseline, BL), along (1, 4, 8, 12, 20 weeks) and 56 weeks after the start of therapy (W56) were collected, used for characterizing the viral load (Figure 11A), liver function (Figure 11B&C) and NK cell numbers (Figure 11D&E) and phenotypes (Chapters 4.1.2, 4.1.3 & 4.1.4). (B) NK cells were sorted by flow cytometry, gating on the CD3-CD4-CD14-CD19-CD56+ population. These sorted NK cells were then analyzed by label-free based LC-MS/MS, using triplicates for each sample. The generated data were processed by MaxQuant and R to get the patient-specific quantification of each protein.
Clinical data regarding (A) Expression of HCV RNA, (B) levels of alanine transferase (ALT), (C) levels of aspartate transaminase (AST) were collected from routine clinical diagnosis. The phenotype on (D) absolute numbers of lymphocytes, (E) absolute numbers of NK cells, and (F) percentage of CD56<sup>dim</sup> NK cells in the HCV patients was measured by flow cytometry. Analysis were done in cooperation with Dr. Julia Hengst (MHH, Hannover).

**4.1.2. NK cell proteomes were altered in chronic HCV patients before and after treatment**

In total, 4867 proteins (Supplementary Table S1) were identified from about 30,000 peptides. To get the most reliable information, I kept 4043 proteins that are robustly identified and quantified in at least 15 out 18 NK cell samples in one group from BL, W56 or healthy. After transforming the LFQ intensities of 4043 proteins into log2 values, I calculated the Pearson correlation factors to evaluate the similarities of all the proteins between the measurements. As shown in Figure 12, the correlation factors vary from 0.93 to 1.00, indicating an overall similarity among all analyzed NK cell samples. As expected, the correlation factors were higher in all technical replicates (black boxes in Figure 12) than in biological replicates either from patients or healthy controls, which indicate the robustness of the proteomic analyses. The donor variance became apparent by comparing correlation factors. NK cell proteome of healthy donors revealed a higher correlation with each other (red box in the upper-left of Figure 12), indicating...
Results

a lower donor variance. In the BL group, the variance was much higher, as three patients are less correlated with each other while the other three patients are similar to each other (red box in the middle of Figure 12). Of note, after treatment, the proteomes of patients’ NK cells are homogenized again as they have higher correlation factors (red box in the lower-right of Figure 12). Interestingly, the proteomes of NK cells from patients before and after treatment were found to have much lower correlations compared to the proteomes of NK cells from healthy donors (blue boxes in Figure 12), indicating that although the NK cell phenotype of cured patients are homogenized, they remain different to healthy controls even one year after therapy.

To reduce the complexity and increase the robustness of the data, I merged the technical replicates by taking median values. Based on these data, principal component analyses were performed to cluster the NK cell proteomes from patients before and after treatment, as well as healthy donors. Similar to the conclusions obtained using correlation factors (Figure 12), Patients before treatment are largely heterogeneous, as three patients are similar to each other and the other three patients are less similar (Figure 13). The patients after treatment are more homogeneous and clustered together. But both groups are completely separated from the healthy donors, suggesting sustained but different alterations in NK cell proteome from chronic HCV patients before and after DAA therapy.

Taken together, these analyses suggested that NK cell proteomes from chronic HCV patients before treatment are heterogeneous and different from healthy donors. Upon Harvoni® treatment, however, donor-variation of NK cell proteomes was minimized but protein expression pattern remained notably different from those of healthy individuals.
Results

Figure 12 | Correlation analyses of NK cell proteomes from healthy individuals and HCV infected patients before and after DAA therapy. Heatmap showing the correlation factors of proteomic data (4043 proteins) of NK cells from HCV patients and healthy donors. Pearson’s method was used for calculating correlation factors. Black boxes indicate the correlation factors of technical replicates, blue boxes show the correlation of patients samples (BL and W56) with healthy donors, and red boxes indicate the inter-individual variance within each group (H, BL and W56).
Results

Figure 13 | Principal component analysis of proteomic data of NK cells from patients and healthy donors. Total proteome data (4043 proteins) were used for PCA analysis, the percentage variance for each principal component is given on the axis.

4.1.3. Determination of significantly regulated proteins

To find out which proteins contributed to the differences between healthy donors and chronic HCV patients before and after DAA treatment, I performed Student’s t-tests for each protein to compare the mean value of its abundance in each sample group, thus comparing BL and W56 NK cell proteomes with healthy donors NK cell proteomes. Based on fold change (difference in log2 values) and p-value from the Student’s t-test I created volcano plots. To analyze the top-regulated proteins (see chapter 4.1.4.1 and chapter 4.1.5.1) and the rescued proteins (see chapter 4.1.4.2), I used a stringent threshold (Level A: log2 value of fold change > 0.2, p-value < 0.01) to determine the significantly regulated proteins (Supplementary Figure S2). To analyze the expression pattern of surface proteins (see chapter 4.1.4.4 and chapter 4.1.5.2) and enrichment of pathways (see chapter 4.1.4.5 and chapter 4.1.5.6), I applied a less stringent threshold (level B: log2 value of fold change > 0.1, p-value < 0.05). The complete list of 4043 proteins regulation information can be seen at Supplementary Table S2. Due to the higher donor variation within the BL group and lower donor variation within the W56 group, there were much less significantly regulated proteins at BL than at W56.

Using Level A threshold, in BL there were 115 proteins significantly down-regulated and 193 proteins significantly up-regulated compared to healthy donors (Figure 14A), while in W56 there were 458 proteins significantly down-regulated and 777 proteins significantly up-regulated (Figure 14C). To visualize the dynamic change of regulation, I combined the information from both volcano plots and integrated the number of significantly up-regulated, down-regulated and
non-regulated proteins. As summarized in Figure 14 B, most of the up-regulated and down-regulated proteins in the BL stayed up- (n=99) or down- (n=163) regulated at W56, respectively. Only 16 downregulated and 30 up-regulated proteins in BL were normalized to the level in healthy individuals in the W56 group. Interestingly, a larger proportion of non-regulated proteins in BL became either down-regulated (n=359) or up-regulated (n=614) in the W56 group, probably as a result of higher variation in BL and lower variation in W56.

Based on the regulation profiles in BL and W56, I classified the significantly regulated proteins into six profile groups (Figure 15). The first group includes the proteins that are down-regulated in chronic HCV-infected patients but rescued to the healthy level by Harvoni® therapy (BL↓ W56≈, 16 proteins), e.g. signal recognition particle 9 kDa protein (SRP9) 173, a protein that is crucial for protein translation and secretion. The second group contains proteins that are enriched in BL but normalized to the healthy level in W56 (BL↑ W56≈, 30 proteins), represented by MX2, which has potent antiviral activity and is regulated by the interferon pathway during HCV infection 174,175. The third profile belongs to those proteins which are down-regulated in both BL and W56 (BL↓ W56↓, 99 proteins), exemplified by the surface marker CD59, which is involved in signaling transduction for T cell and NK cell activation 176,177. The next group contained the proteins having a sustained up-regulation profile (BL↑ W56↑, 163 proteins), illustrated by FYB, which promotes NK cell degranulation and cytokine production 79,178. The last two groups are down- (BL≈ W56↓, 359 proteins) or up-regulated (BL≈ W56↑, 614 proteins) only in W56, with

Figure 14 | Determination of significantly regulated proteins. The volcano plots were generated based on the fold change of BL (A) and W56 (C) in comparison to healthy individuals and the corresponding p values. Using the stringent threshold (level A: p-value<0.01, log2 value of fold change>0.2), the significantly dysregulated proteins are coded with different colors. (B) Sankey diagram integrates the information of significantly regulated proteins in NK cells from BL and W56 patients compared to healthy donors.
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CD47 and Cyclin L2 (CCNL2) as examples, respectively. CD47 regulates systemic NK cell activation and exhaustion in the tumor microenvironment and CCNL2 splices pre-mRNA and induce cell death and might be manipulated to promote virus replication. The majority (68%) of identified proteins are not significantly regulated in both groups (BL≈ W56≈).

Taken together, these analyses suggest that the significantly regulated proteins can be classified into six expression pattern profiles in the NK cell proteomes from HCV-infected patients before (BL) and after (W56) Harvoni treatment.

![Pie Chart showing distinct expression profiles of proteins in patients’ and healthy donor NK cells. Only 46 protein regulations are rescued in therapy (group 1 and 2; 16 + 30 proteins) including protein translation / secretion (SRP9) and interferon signaling (MX2). Non-rescued protein regulations (four groups) indicate impaired (CD59) and trained NK cell immunity (FYB). Up to 24% of the NK cell proteome (9% + 15%) indicates a switch of the NK cell phenotype including the innate immunity checkpoint CD47 and other known viral targets of the cell cycle (CCNL2).](image)

**Figure 15 | Six groups of protein regulation profiles before and after therapy.** Pie Chart showing distinct expression profiles of proteins in patients’ and healthy donor NK cells. Only 46 protein regulations are rescued in therapy (group 1 and 2; 16 + 30 proteins) including protein translation / secretion (SRP9) and interferon signaling (MX2). Non-rescued protein regulations (four groups) indicate impaired (CD59) and trained NK cell immunity (FYB). Up to 24% of the NK cell proteome (9% + 15%) indicates a switch of the NK cell phenotype including the innate immunity checkpoint CD47 and other known viral targets of the cell cycle (CCNL2).

**4.1.4. Molecular phenotype of NK cells in chronic HCV patients before DAA therapy**

4.1.4.1. Identification of top-dysregulated NK cell proteins

In NK cells of chronic HCV patients before DAA therapy (BL, baseline), stringent threshold (level A: p-value < 0.01, log2(BL/H) > 0.2) identified 115 proteins as significantly down-regulated and 193 proteins as significantly up-regulated as compared to healthy controls (see chapter 4.1.3). Some of these proteins were already reported to be related to HCV infection, e.g.
CD59 could be incorporated into HCV virions and MX2 was reported to be an interaction partner of HCV NS5A protein and inhibit HCV replication. However, most of the significantly dysregulated proteins here being reported to be dysregulated in HCV infection for the first time, and therefore might be novel candidate proteins involved in regulating chronic HCV infections.

To analyze the NK cell phenotypes that are affected by chronic HCV infections the most, I focused on the top 20 down-regulated (Table 7) and top 20 up-regulated proteins in BL patients (Table 8). Down-regulation of NK cell-derived proteins in BL patients might indicate inhibited or dysregulated NK cell function. Here, amongst others, I found cytoskeleton proteins (TUBB8, TPM3 and DYNLL2), proteins associated with the Golgi apparatus (CD59, ARF5, CORO7 and SMPD4) and mitochondrion proteins (CISD2, GTPBP3, COX6B1, FXN and UQCR10) to be down-regulated in NK cells from BL patients. The up-regulation of NK cells derived proteins suggests these proteins are potentially involved in the antiviral immunity against HCV infection. In this study I found the proteins involved in defense response (MX2 and MICU1), cytoskeletal proteins (ACTBL2 and POTEE) and histone proteins (HIST1H2BK, HIST1H1E) to be up-regulated in NK cell from BL patients. Interestingly, out of these forty top-dysregulated proteins in BL patients, only six proteins (TUBB8, GTPBP3, TXNL4A, CORO7, COX6B1 and MX2) were normalized in W56 patients to the level of healthy controls.

In summary, these data suggest that molecules of the cytoskeleton, Golgi apparatus and mitochondrion were enriched as part of the top down-regulated proteins, while defense response proteins and histone proteins were mostly detected in the top up-regulated proteins in the NK cells of BL patients.
### Table 7 | Top 20 down-regulated proteins in NK cells of BL patients. Proteins rescued in W56 patients to the healthy level are labeled blue.

<table>
<thead>
<tr>
<th>ID</th>
<th>Protein.names</th>
<th>Gene.names</th>
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<th>BL: Level A</th>
<th>Log2 (W56/H)</th>
<th>W56: Level A</th>
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<td>Q3ZCM7</td>
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<td>TUBB8</td>
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<td>+</td>
<td>-0.82</td>
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<tr>
<td>P05976</td>
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<td>MYL1</td>
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<td>-2.01</td>
<td>+</td>
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<td>E9PNW4</td>
<td>CD59 glycoprotein</td>
<td>CD59</td>
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<td>+</td>
<td>-2.36</td>
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<tr>
<td>Q59GN2</td>
<td>Putative 60S ribosomal protein L39-like 5</td>
<td>RPL39P5</td>
<td>-3.07</td>
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<td>A0A087WW US</td>
<td>Tropomyosin-alpha-3 chain</td>
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<td>Q969Y2</td>
<td>tRNA modification GTPase GTPBP3, mitochondrial</td>
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<td>A0A0A0MT3 2</td>
<td>Lysosomal acid lipase/cholesterol ester hydrolase</td>
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<td>C9JAX1</td>
<td>Frataxin, mitochondrial</td>
<td>FXN</td>
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<td>D6RBQ9</td>
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<td>HNRNP D</td>
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<td>Q8NKX1</td>
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<td>Q8TB03</td>
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<td>-1.80</td>
<td>+</td>
<td>-1.16</td>
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### Table 8 | Top 20 up-regulated proteins in NK cells of BL patients. Proteins normalized in W56 patients to the healthy level are labeled blue.

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<tr>
<th>ID</th>
<th>Protein.names</th>
<th>Gene.names</th>
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<th>Log2 (W56/H)</th>
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<td>Beta-actin-like protein 2</td>
<td>ACTBL2</td>
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<td>O60814</td>
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<td>HIST1H2BK</td>
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<td>P0DN24</td>
<td>Uncharacterized protein C3orf86</td>
<td>C3orf86</td>
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<td>O00481</td>
<td>Butyrophilin subfamily 3 member A1</td>
<td>BTN3A1</td>
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<td>H0YIV9</td>
<td>Glutaminyl-tRNA(Gln) amidotransferase subunit C, mitochondrial</td>
<td>GATC</td>
<td>3.54</td>
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<td>O95749</td>
<td>Geranylgeranyl pyrophosphate synthase</td>
<td>GGPS1</td>
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<td>+</td>
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<td>D6RIB6</td>
<td>Macrophage erythroblast attacher</td>
<td>MAEA</td>
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<td>Q13416</td>
<td>Origin recognition complex subunit 2</td>
<td>ORC2</td>
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<td>P10412</td>
<td>Histone H1.4</td>
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<td>P20592</td>
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## Results

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<td>Beta-glucuronidase</td>
<td>GUSB</td>
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<td>Q9H299</td>
<td>SH3 domain-binding glutamic acid-rich-like protein 3</td>
<td>SH3BGR L3</td>
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<td>+</td>
<td>2.16</td>
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<td>F6XEV2</td>
<td>Calcium uptake protein 1, mitochondrial</td>
<td>MICU1</td>
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<td>Q96T49</td>
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<td>SUMF2</td>
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</table>

### 4.1.4.2. Rescued proteins could reveal NK cell functions controlled by HCV

To find out by which mechanism HCV dysregulate NK cell immunity during chronic infection, I next focus on the group of proteins that were **down-regulated** in NK cells of BL patients but rescued in W56 NK cells. Whereas proteins up-regulated only in BL (BL↑ W56↓, 30 proteins, Supplementary Table S3) might be induced by infection-stimulated responses (e.g. MX2, and PTPN1), proteins down-regulated in BL but rescued by DAA therapy (BL↓ W56↑, 16 proteins, **Table 9**) might be NK cells responses controlled by HCV infection.

Of these proteins down-regulated in BL and rescued in W56, TXNL4A, DDX19A, ZRANB2, LAS1L, SRP9 are RNA binding proteins that could interfere the translation and replication of HCV or promote the translation of antiviral proteins. Therefore, down-regulation of these RNA binding proteins might be a mechanism for HCV to control NK cell antiviral immunity. Additionally, TUBB8, GTPBP3, ARHGAP31 and CORO7 are GTPase-related proteins that might facilitate HCV replication and participate in the polarization of cytolytic effectors and cell migration. Down-regulation of these proteins could be either a response of NK cells to inhibit HCV replication or an immune evasion strategy of HCV to impair NK cell immunity by interfering with migration and degranulation. NPC2 promotes post-lysosomal export of cholesterol and transport cholesterol between endosome and lysosome is essential for HCV replication. Accordingly, the down-regulation of NPC2 in BL could be used by NK cells to block the HCV life cycle. Of note, TXNL4A, TUBB8, GTPBP3, CORO7 and COX6B1 were the top 20 down-regulated proteins in the BL patients but were rescued in the W56 patients,
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suggesting these proteins were under targeted by chronic HCV infection but were reversed by the DAA treatment.

Taken together, these rescued proteins suggest that the NK cells in BL patients were responding to the HCV infection by up-regulating ISGs and inhibit cholesterol transport, but their function in RNA-binding and GTPase activity were inhibited by chronic HCV infection.

Table 9 | Proteins down-regulated in BL but rescued in W56 to the level of healthy controls. RNA binding proteins are shown in green, proteins related to GTPases are shown in red. The proteins that were the top-regulated in BL patients are shown in bold.

<table>
<thead>
<tr>
<th>IDs</th>
<th>Protein names</th>
<th>Gene names</th>
<th>Log2 (BL/H)</th>
<th>BL: Level A</th>
<th>Log2 (W56/H)</th>
<th>W56: Level A</th>
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<td>P83876</td>
<td>Thioredoxin-like protein 4A</td>
<td>TXNL4A</td>
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<td>+</td>
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<td>F6QDS0</td>
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<td>Zinc finger Ran-binding domain-containing protein 2</td>
<td>ZRANB2</td>
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<td>Q9Y4W2</td>
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<td>TUBB8</td>
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<td>+</td>
<td>-0.82</td>
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4.1.4.3. Chronic HCV infection targets degranulation but not cytotoxic molecules.

Lytic granules contain perforin and granzymes that are crucial for NK cell cytotoxicity. As the cytotoxicity of NK cells in chronic HCV patients was reported to be impaired, one could speculate that the contents of lytic granules might be affected. Therefore, I evaluated the expression of components of lytic granules in the NK cell proteomes from BL and W56 patients. As shown in Figure 16, the granzymes and perforin have no significantly different expression in the NK cells from patients, both at BL and W56, in comparison with healthy donors. Granulysin, which induces target cell apoptosis through increasing intracellular calcium and
decreasing intracellular potassium, was higher only in the W56 sample when compared to healthy donors. However, the expression of CD107a (LAMP1), a molecule that promotes the delivery of perforin and granzymes into target cells, was found significantly down-regulated both at BL and W56. These data suggest that the degranulation, but not the cytotoxic proteins in NK cells was impaired by chronic HCV infection in both BL and W56 patients.

Figure 16 | Expression patterns of cytolytic molecules and CD107a. Box and jitter plots show the expression of cytolytic proteins in healthy controls (H), HCV patients before treatment (BL) and after treatment (W56). Each point indicates the median value of three technical replicates. Box plots show the median and quantiles for each group. The Stars indicate significant differences of protein expression determined by Student’s t-test: *p < 0.05, **p < 0.01, ***p < 0.001.
Results

4.1.4.4. Surface receptors profiles suggest reduced NK cell responsiveness

Since various studies showed that chronic HCV infection can affect the surface expression of activating and inhibitory receptors\textsuperscript{121,130}, the next question I asked was whether the protein abundances of receptors in NK cells might be altered upon HCV infection. For that, I sorted out all the proteins that are potentially expressed on the cell surface based on the in silico human surfaceome\textsuperscript{190} and checked their expression patterns using the less stringent threshold (Level B significance, see Figure 17A). In total, 108 identified proteins in this study (Supplementary Table S4) were classified as surface proteins and, surprisingly, 30 of these proteins were significantly dysregulated in NK cells of BL patients. These include activating and inhibitory receptors that are already studied in NK cell immunity, as well as surface proteins with elusive immune functions in NK cells.

Of these activating receptors, CD59 glycoprotein, protein-tyrosine-phosphatase (PTPRC, CD45), and bone marrow stromal antigen 2 (BST2, CD317) were down-regulated in the NK cells of chronic HCV patients (BL); butyrophilin subfamily 3 member A1 (BTN3A1, CD277) and DNAM-1 were up-regulated in the NK cells of BL patients. The integrin alpha-L (CD11a, ITGAL) was down-regulated while integrin beta (ITGB2, CD18) and integrin beta-7 (ITGB7) were up-regulated in the NK cells of BL patients. Of note, all of the identified inhibitory receptors, including CMRF35-like molecule 8 (CMRF35H, CD300a), G-protein coupled receptor 56 (GPR56) and leukocyte-associated immunoglobulin-like receptor 1 (LAIR1, CD305), were up-regulated in the NK cells of BL patients. Of the receptors that contain both activating and inhibitory functions, SLAM family member 7 (SLMF7, CD319) was down-regulated, while hepatitis A virus cellular receptor 2 (HAVCR2, Tim-3), CD48 and sialic acid-binding Ig-like lectin 7 (SIGLEC7, CD328) were down-regulated in NK cells of BL patients.

Taken together, most of the found dysregulated activating receptors were down-regulated and all identified inhibitory receptors were up-regulated in the NK cells of BL patients, suggesting that NK cell function is impaired in the chronically HCV-infected patients.
Table 10 | Dysregulated surface receptors in NK cells of BL patients.

Surface proteins that have significantly (less stringent threshold, Level B) altered expression in chronic HCV infected patients compared to healthy donors. Activating receptors are labeled red, inhibitory receptors are labeled light green, and receptors that have both activating and inhibitory functions are labeled yellow, integrins are label blue.

<table>
<thead>
<tr>
<th>ID</th>
<th>Proteins</th>
<th>Genes</th>
<th>Reported functions</th>
<th>Log2 (BL/H)</th>
<th>BL: level B</th>
<th>Log2 (W56/H)</th>
<th>W56: level B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q10589</td>
<td>Bone marrow stromal antigen 2</td>
<td>BST2 (CD317)</td>
<td>IFN-induced antiviral host restriction factor, moderately restricts HCV production and release from HuH-7.5 hepatocytes (^\text{191}).</td>
<td>-1.16</td>
<td>+</td>
<td>-0.66</td>
<td>+</td>
</tr>
<tr>
<td>E9PNW 4</td>
<td>CD59 glycoprotein</td>
<td>CD59</td>
<td>Incorporated into HCV virions and in turn, protect HCV from ADCML (^\text{192}), Co-receptor of NKP46 and Nkp30 and activates NCRs mediated-cytotoxicity (^\text{177}).</td>
<td>-3.07</td>
<td>+</td>
<td>-2.36</td>
<td>+</td>
</tr>
<tr>
<td>A0A0A0MT22</td>
<td>Protein-tyrosine-phosphatase</td>
<td>PTPRC (CD45)</td>
<td>Protein tyrosine-protein phosphatase required for T-cell activation through the antigen receptor. Regulate DNAM1-mediated killing activity (^\text{192}).</td>
<td>-0.51</td>
<td>+</td>
<td>-0.60</td>
<td>+</td>
</tr>
<tr>
<td>Q15762</td>
<td>CD226 antigen</td>
<td>DNAM-1 (CD226)</td>
<td>Regulates NK cell antitumor responses (^\text{193,194}).</td>
<td>0.73</td>
<td>+</td>
<td>-0.50</td>
<td>+</td>
</tr>
<tr>
<td>OO0481</td>
<td>Butyrophilin subfamily 3 member A1</td>
<td>BTN3A1 (CD277)</td>
<td>A co-regulator of the immune signal in T and NK cells (^\text{195}).</td>
<td>4.27</td>
<td></td>
<td>5.39</td>
<td>+</td>
</tr>
<tr>
<td>Q9UGN 4</td>
<td>CMRF35-like molecule 8</td>
<td>CD300a</td>
<td>Contributes to the down-regulation of cytolytic activity in natural killer (NK) cells (^\text{196,197}). By associating with the tetraspanin CD81 negatively regulates immediate effector functions, including production of inflammatory cytokines and cytolytic proteins, degranulation, and target cell killing (^\text{198}).</td>
<td>0.45</td>
<td>+</td>
<td>0.59</td>
<td>+</td>
</tr>
<tr>
<td>Q9Y653</td>
<td>G-protein coupled receptor 56</td>
<td>GPR56 (ADGRG1)</td>
<td></td>
<td>0.68</td>
<td>+</td>
<td>0.62</td>
<td>+</td>
</tr>
<tr>
<td>A0A0G2JNK8</td>
<td>Leukocyte-associated immunoglobulin in-like receptor 1</td>
<td>LAIR1 (CD305)</td>
<td>An inhibitory receptor that plays a constitutive negative regulatory role on cytolytic function of natural killer (NK) cells, B-cells and T-cells (^\text{199}).</td>
<td>1.07</td>
<td>+</td>
<td>1.35</td>
<td>+</td>
</tr>
<tr>
<td>Q9Y286</td>
<td>Sialic acid-binding Ig-like lectin 7</td>
<td>SIGLEC7 (CD328)</td>
<td>Mediates inhibition of natural killer cells cytotoxicity. Expression of Siglec-7 was significantly decreased on NK cells from HCV-infected and HBV-infected patients (^\text{200}).</td>
<td>-0.88</td>
<td>+</td>
<td>-0.88</td>
<td>+</td>
</tr>
<tr>
<td>A0A087X1S7</td>
<td>CD48 antigen</td>
<td>CD48</td>
<td>As both an activating and inhibitory receptor, either promoting or inhibiting target lysis on NK cells (^\text{201}).</td>
<td>-0.90</td>
<td>+</td>
<td>-1.07</td>
<td>+</td>
</tr>
<tr>
<td>ESRHN 3</td>
<td>Hepatitis A virus cellular receptor 2</td>
<td>HAVCR2 (Tim-3)</td>
<td>Regulate NK cells both positively and negatively, depending on the ligand presented by the target cell (^\text{202,203}).</td>
<td>-1.11</td>
<td>+</td>
<td>-0.10</td>
<td>+</td>
</tr>
<tr>
<td>Q9NQ2 5</td>
<td>SLAM family member 7</td>
<td>SLAMF7 (CD319)</td>
<td>Positively regulates NK cell functions by a mechanism dependent on phosphorylated SH2D1B. However, in the absence of SH2D1B, inhibits NK cell function (^\text{146,204}).</td>
<td>0.46</td>
<td>+</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>P32942</td>
<td>Intercellular adhesion molecule 3</td>
<td>ICAM-3 (CD50)</td>
<td>Ligand for the leukocyte adhesion protein LFA-1, contributes to apoptotic neutrophil phagocytosis by macrophages (^\text{205}).</td>
<td>0.20</td>
<td>+</td>
<td>0.59</td>
<td>+</td>
</tr>
<tr>
<td>D3DSM 0</td>
<td>Integrin beta</td>
<td>ITGB2 (CD18)</td>
<td>Required for optimal NK-cell development and function (^\text{206}).</td>
<td>0.56</td>
<td>+</td>
<td>0.58</td>
<td>+</td>
</tr>
<tr>
<td>P26010</td>
<td>Integrin beta-7</td>
<td>ITGB7</td>
<td>An adhesion molecule that mediates lymphocyte migration and homing (^\text{207}).</td>
<td>0.77</td>
<td>+</td>
<td>0.49</td>
<td>+</td>
</tr>
<tr>
<td>P20701</td>
<td>Integrin alpha-L</td>
<td>ITGAL (CD11a)</td>
<td>Involved in a variety of immune phenomena including leukocyte-endothelial cell interaction. Contributes to NK cell cytotoxicity (^\text{54}).</td>
<td>-0.57</td>
<td>+</td>
<td>-0.72</td>
<td>+</td>
</tr>
</tbody>
</table>
4.1.4.5. Enrichment analyses revealed impaired cellular NK cell pathways

To identify the differentially regulated biological processes in the NK cells of chronic HCV patients before treatment (BL), I performed systematic pathway enrichment assay on the whole proteome data based on the method adapted from Pan et al. 167. According to the level A (log2 value of fold change > 0.2, p-value < 0.01) and level B (log2 value of fold change > 0.1, p-value < 0.05) significant regulation, all the proteins were divided into 5 sections (Figure 17A): down-regulated in level A (Q1), down-regulated only in level B (Q2), not significantly regulated (Q3), up-regulated only in level B (Q4), and up-regulated in level A (Q5). In Perseus the proteins were annotated with gene ontology (GO) terms: including “KEGG pathways” and GO “Biological Processes” (GOBP). In each section every item was analyzed by Fisher’s Exact Test 208 to determine the direction of enrichment and whether it is over-represented (marked with “+”) or under-represented (marked with “-”). Then the significantly (p<0.05) enriched items were selected and are listed in Supplementary Table S5A (“KEGG pathways” for BL vs H), supplementary table S5B (“Biological processes” for BL vs H). Then I selected the items related to NK cell biology and created a heatmap and hierarchical clustering based on the transformed p-values. Therefore, these data can provide an overview of the regulation of pathways and biological processes in NK cells of BL patients compared to healthy donors and extract the immunity-related phenotypes.

Expression of proteins in KEGG pathways, including the MAPK signaling pathway, and proteasome are enriched in Q4, higher in BL than in healthy donors. Proteins involved in oxidative phosphorylation, chemokine-mediated pathway, and leukocyte transendothelial migration are enriched in Q1 and Q2, lower in BL patients than in healthy donors (Figure 17B). In the case of GOBP, interestingly only evasion by virus of host immune response are enriched in Q5 (higher in BL), while the functions indicating immunity responses, e.g. natural killer cell-mediated cytotoxicity, intracellular receptor-mediated signaling pathways, positive regulation of chemokine secretion, immune effector process are all enriched in Q1 and Q2, lower in BL patients than in healthy NK cells (Figure 17C).

In summary, these analyses suggest that chronic HCV infection stimulated the MAPK signaling pathway, proteasome, and evasion by virus of host immune response, but impaired several immune effector-related pathways in NK cells of chronically HCV infected patients.
Figure 17 | Pathway analyses of the NK cell proteome in chronic HCV patients.

(A) Comparing NK cell proteome of chronic HCV patients (BL) and healthy donors (H), 5 sections were determined by level A (log2 value of fold change > 0.2, p-value < 0.01) and level B (log2 value of fold change > 0.1, p-value < 0.05) significant regulations in volcano plot: down-regulated in level A (Q1), down-regulated in level B (Q2), not significantly regulated (Q3), up-regulated in level B (Q4), and up-regulated in level A (Q5). These quantiles were evaluated separately for enrichment assay of KEGG pathways (B) and gene ontology biological processes (GOBP) (C). The proteins were clustered according to the p-values. “+” and “-” are used to indicate the direction of enrichment (“+” for over-representation, “-” for under-representation). The “0” indicates no difference. Significant enrichment (p < 0.05) is visualized in yellow/red for over-represented and in light blue/dark blue for under-represented pathways, whereas non-significant enrichment (p>0.05) is displayed in green.

4.1.5. Molecular phenotype of NK cells in the HCV patients after DAA therapy

In NK cells of HCV patients 56 weeks after the start of DAA therapy, analyses using stringent threshold (level A: p-value < 0.01, log2(BL/H) > 0.2) identified 458 proteins as significantly down-regulated and 777 proteins as significantly up-regulated (see chapter 4.1.3). Interestingly, there were more proteins dysregulated in NK cells of cured HCV patients (W56) than in patients before treatment (BL). The reason behind might be the donor variation in NK cells of W56 patients was lower than in NK cells of BL patients, resulting in the shift of proteins from non-regulated in BL to significantly dysregulation in W56. This study identifies 359 proteins that shifted from non-regulated in BL to significantly down-regulated in W56 (BL  W56↓) and 614 proteins that shifted from non-regulated in BL to significantly up-regulated in W56 (BL  W56↑).
Results

4.1.5.1. Identification of top-dysregulated NK cell proteins

To answer the question whether NK cell immunity was rescued in the cured HCV patients, I analyzed the alterations of NK cell function in cured patients through characterizing the top 20 down-regulated proteins (Table 11) and top 20 up-regulated proteins (Table 12) based on the fold changes in the NK cells from HCV patients 56 weeks after DAA treatment (W56) compared to NK cells from healthy donors.

Interestingly, most of the top-dysregulated proteins in NK cells of W56 patients were already the top dysregulated in NK cells of chronic HCV patients before DAA treatment (BL), indicative for sustained regulation of NK cell proteomes upon HCV infection even after viral clearance. Proteins that are sustained down-regulated are the cytoskeletal proteins (TPM3 and DYNLL2), the Golgi apparatus proteins (CD59, ARF5 and SMPD4) and mitochondrial proteins (CISD2, FXN and UQCR10).

Table 11 | Top 20 down-regulated proteins in NK cells of W56 patients. The proteins that were also top 20 down-regulated in BL are labeled green.

<table>
<thead>
<tr>
<th>IDs</th>
<th>Proteins</th>
<th>Genes</th>
<th>Log2 (BL/H)</th>
<th>BL: Level A</th>
<th>Log2 (W56/H)</th>
<th>W56: Level A</th>
</tr>
</thead>
<tbody>
<tr>
<td>P05204</td>
<td>Non-histone chromosomal protein HMG-17</td>
<td>HMGN2</td>
<td>-1.65</td>
<td>+</td>
<td>-3.68</td>
<td>+</td>
</tr>
<tr>
<td>P60903</td>
<td>Protein S100-A10</td>
<td>S100A10</td>
<td>-2.50</td>
<td>+</td>
<td>-3.35</td>
<td>+</td>
</tr>
<tr>
<td>A0A087WWU8</td>
<td>Tropomyosin alpha-3 chain</td>
<td>TPM3</td>
<td>-2.73</td>
<td>+</td>
<td>-2.80</td>
<td>+</td>
</tr>
<tr>
<td>C9JAX1</td>
<td>Frataxin, mitochondrial</td>
<td>FXN</td>
<td>-2.14</td>
<td>+</td>
<td>-2.80</td>
<td>+</td>
</tr>
<tr>
<td>Q9GZL7</td>
<td>Ribosome biogenesis protein WDR12</td>
<td>WDR12</td>
<td>-0.90</td>
<td>+</td>
<td>-2.71</td>
<td>+</td>
</tr>
<tr>
<td>Q96FJ2</td>
<td>Dynein light chain 2, cytoplasmic</td>
<td>DYNLL2</td>
<td>-2.25</td>
<td>+</td>
<td>-2.70</td>
<td>+</td>
</tr>
<tr>
<td>Q9UDW1</td>
<td>Cytochrome b-c1 complex subunit 9</td>
<td>UQCR10</td>
<td>-1.92</td>
<td>+</td>
<td>-2.69</td>
<td>+</td>
</tr>
<tr>
<td>Q5SRN5</td>
<td>HLA class I histocompatibility antigen, A-3 alpha chain</td>
<td>HLA-A</td>
<td>-2.42</td>
<td>+</td>
<td>-2.50</td>
<td>+</td>
</tr>
<tr>
<td>P04259</td>
<td>Keratin, type II cytoskeletal 6B</td>
<td>KRT6B</td>
<td>-0.53</td>
<td>+</td>
<td>-2.42</td>
<td>+</td>
</tr>
<tr>
<td>P17096</td>
<td>High mobility group protein HMG-I/HMG-Y</td>
<td>HMGA1</td>
<td>-0.96</td>
<td>+</td>
<td>-2.36</td>
<td>+</td>
</tr>
<tr>
<td>E9PNW4</td>
<td>CD59 glycoprotein</td>
<td>CD59</td>
<td>-3.07</td>
<td>+</td>
<td>-2.36</td>
<td>+</td>
</tr>
<tr>
<td>D6RBQ9</td>
<td>Heterogeneous nuclear ribonucleoprotein D0</td>
<td>HNRPD</td>
<td>-2.00</td>
<td>+</td>
<td>-2.35</td>
<td>+</td>
</tr>
<tr>
<td>P84085</td>
<td>ADP-ribosylation factor 5</td>
<td>ARF5</td>
<td>-2.18</td>
<td>+</td>
<td>-2.32</td>
<td>+</td>
</tr>
<tr>
<td>Q92783</td>
<td>Signal transducing adaptor molecule 1</td>
<td>STAM1</td>
<td>-1.39</td>
<td>+</td>
<td>-2.15</td>
<td>+</td>
</tr>
<tr>
<td>Q8NGH1</td>
<td>Tetraspanin-14</td>
<td>TSPAN14</td>
<td>-1.42</td>
<td>+</td>
<td>-2.10</td>
<td>+</td>
</tr>
<tr>
<td>O00629</td>
<td>Importin subunit alpha-3</td>
<td>KPNA4</td>
<td>-1.72</td>
<td>+</td>
<td>-2.09</td>
<td>+</td>
</tr>
<tr>
<td>O75563</td>
<td>Src kinase-associated phosphoprotein 2</td>
<td>SKAP2</td>
<td>-0.45</td>
<td>+</td>
<td>-2.09</td>
<td>+</td>
</tr>
<tr>
<td>A0A0A0MT32</td>
<td>Lysosomal acid lipase/cholesteryl ester hydrolase</td>
<td>LIPA</td>
<td>-2.37</td>
<td>+</td>
<td>-2.08</td>
<td>+</td>
</tr>
<tr>
<td>Q96CW5</td>
<td>Gamma-tubulin complex component 3</td>
<td>TUBGCP3</td>
<td>-1.09</td>
<td>+</td>
<td>-2.07</td>
<td>+</td>
</tr>
<tr>
<td>P68871</td>
<td>Hemoglobin subunit beta</td>
<td>HBB</td>
<td>-0.29</td>
<td>+</td>
<td>-2.05</td>
<td>+</td>
</tr>
</tbody>
</table>
### Results

Table 12 | Top 20 up-regulated proteins in NK cells of W56 patients. The proteins that were also top 20 up-regulated in BL are labeled red.

<table>
<thead>
<tr>
<th>IDs</th>
<th>Protein.names</th>
<th>Gene.names</th>
<th>Log2 (BL/H)</th>
<th>BL: Level A</th>
<th>Log2 (W56/H)</th>
<th>W56: Level A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q562R1</td>
<td>Beta-actin-like protein 2</td>
<td>ACTBL2</td>
<td>9.44</td>
<td>+</td>
<td>9.35</td>
<td>+</td>
</tr>
<tr>
<td>Q6S8J3</td>
<td>POTE ankyrin domain family member E</td>
<td>POTEE:POTEF</td>
<td>1.85</td>
<td>+</td>
<td>8.27</td>
<td>+</td>
</tr>
<tr>
<td>P0DN24</td>
<td>Uncharacterized protein C3orf86</td>
<td>C3orf86</td>
<td>6.21</td>
<td>+</td>
<td>7.68</td>
<td>+</td>
</tr>
<tr>
<td>O60814</td>
<td>Histone H2B type 1-K</td>
<td>HIST1H2BK</td>
<td>6.36</td>
<td>+</td>
<td>7.32</td>
<td>+</td>
</tr>
<tr>
<td>O00481</td>
<td>Butyrophilin subfamily 3 member A1</td>
<td>BTN3A1</td>
<td>4.27</td>
<td>+</td>
<td>5.39</td>
<td>+</td>
</tr>
<tr>
<td>Q93079</td>
<td>Histone H2B type 1-H</td>
<td>HIST1H2BH</td>
<td>2.49</td>
<td>+</td>
<td>4.62</td>
<td>+</td>
</tr>
<tr>
<td>P5S876</td>
<td>Histone H2B type 1-D</td>
<td>HIST1H2BD</td>
<td>2.43</td>
<td>+</td>
<td>4.59</td>
<td>+</td>
</tr>
<tr>
<td>Q6DRA6</td>
<td>Putative histone H2B type 2-D</td>
<td>HIST2H2BD</td>
<td>0.37</td>
<td>+</td>
<td>3.63</td>
<td>+</td>
</tr>
<tr>
<td>O95749</td>
<td>Geranylgeranyl pyrophosphate synthase</td>
<td>GGPS1</td>
<td>3.28</td>
<td>+</td>
<td>3.34</td>
<td>+</td>
</tr>
<tr>
<td>P62807</td>
<td>Histone H2B type 1-C/E/F/G/I</td>
<td>HIST1H2BC</td>
<td>1.65</td>
<td>+</td>
<td>3.26</td>
<td>+</td>
</tr>
<tr>
<td>P13051</td>
<td>Uracil-DNA glycosylase</td>
<td>UNG</td>
<td>1.08</td>
<td>+</td>
<td>3.09</td>
<td>+</td>
</tr>
<tr>
<td>Q15042</td>
<td>Rab3 GTPase-activating protein catalytic subunit</td>
<td>RAB3GAP1</td>
<td>1.35</td>
<td>+</td>
<td>3.01</td>
<td>+</td>
</tr>
<tr>
<td>Q969Q0</td>
<td>60S ribosomal protein L36a-like</td>
<td>RPL36AL</td>
<td>1.42</td>
<td>+</td>
<td>2.93</td>
<td>+</td>
</tr>
<tr>
<td>H0YIV9</td>
<td>Glutamyl-tRNA(Gln) amidotransferase subunit C, mitochondrial</td>
<td>GATC</td>
<td>3.54</td>
<td>+</td>
<td>2.81</td>
<td>+</td>
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<tr>
<td>Q13136</td>
<td>Liprin-alpha-1</td>
<td>PPFIA1</td>
<td>1.67</td>
<td>+</td>
<td>2.64</td>
<td>+</td>
</tr>
<tr>
<td>H3BUJ7</td>
<td>Transcription factor E4F1</td>
<td>E4F1</td>
<td>1.49</td>
<td>+</td>
<td>2.60</td>
<td>+</td>
</tr>
<tr>
<td>D6RB6</td>
<td>Macrophage erythroblast attacher</td>
<td>MAEA</td>
<td>2.37</td>
<td>+</td>
<td>2.36</td>
<td>+</td>
</tr>
<tr>
<td>P08236</td>
<td>Beta-glucuronidase</td>
<td>GUSB</td>
<td>1.83</td>
<td>+</td>
<td>2.36</td>
<td>+</td>
</tr>
<tr>
<td>Q9H299</td>
<td>SH3 domain-binding glutamic acid-rich-like protein 3</td>
<td>SH3BGR3L</td>
<td>1.83</td>
<td>+</td>
<td>2.16</td>
<td>+</td>
</tr>
<tr>
<td>Q29865</td>
<td>HLA class I histocompatibility antigen, Cw-18 alpha chain</td>
<td>HLA-C</td>
<td>2.45</td>
<td>+</td>
<td>2.12</td>
<td>+</td>
</tr>
</tbody>
</table>

Additionally, other proteins (e.g. HMGN2, STAM1) were also down-regulated in NK cells of BL patients but their expression was further shifted to the top 20 down-regulated proteins only in the NK cells from W56 patients. HMGN2 has been reported to exhibit anti-tumor and anti-virus activity\(^ {177,209}\) and may play a role in NK cell antiviral immunity. STAM1 contains an SH3 domain and an immunoreceptor tyrosine-based activation motif (ITAM) and is associated with Jak2 and Jak3 for the regulation of cell growth\(^ {210}\) and could be involved in the NK cell signaling pathways. Additionally, STAM1, together with homolog STAM2 that is also shifted to significantly downregulated in NK cells of W56 patients, are involved in the trafficking of cargo proteins to lysosome\(^ {211}\). Therefore, STAM1 and STAM2 may also participate in the trafficking of lytic granules in NK cells.

Notably, four proteins were not dysregulated in NK cells of BL patients but shifted to be the top down-regulated proteins in NK cells of W56 patients: cytoskeleton constituent (KRT6B), DNA binding protein (HMGA1), signaling adaptor protein (SKAP2) and oxygen transport protein (HBB). SKAP2 has been reported to interact with ADAP to regulate integrin-mediated adhesion
Results

in T cells\textsuperscript{212,213} and therefore may also be involved in NK cell immunity. The sustained up-regulated proteins include cytoskeletal proteins (ACTBL2 and POTEE) and histone proteins (HIST1H2BK, HIST1H1E). Interestingly, another three histone proteins (HIST1H2BD, HIST2H2BD, HIST1H2BC) were not dysregulated in BL but shifted to the top up-regulated proteins list.

Taken together, the cytoskeletal proteins in the NK cells of DAA-cured HCV patients were found both up- and down-regulated, which effect on NK cell immunity is still elusive. Histone proteins were sustained up-regulated, while Golgi apparatus and mitochondrial proteins, as well as proteins that have antiviral and adaptor activity, were sustained down-regulated in the cured patients.

4.1.5.2. Surface receptors profiles indicate a highly distinct trained NK cell immunity

The Chapter 4.1.4.4 has shown that most of the found dysregulated activating receptors were down-regulated and all identified inhibitory receptors were up-regulated in the NK cells of BL patients. Subsequently, I focused on the expression of surface markers in the W56 patients (Table 13) to evaluate whether NK cell functions were recovered. Most of the dysregulated receptors were persistently dysregulated in NK cells of W56 patients, indicating the long term effects of chronic HCV infection even after viral clearance. Sustained up-regulation of the activating receptors (BTN3A1) and integrins (ITGB2 and ITGB7) suggests trained immunity in the NK cells of cured patients. Sustained down-regulation of activating receptors (CD59, PTPRC and CD317) and integrin (ITGAL), as well as sustained up-regulation of inhibitory receptors (CD300a, GPR56 and LAIR1), support impaired immunity in the NK cells of cured patients.

Table 13 | Dysegulated NK cell surface proteins. Surface proteins that have significantly (less stringent threshold, Level B) altered expression in chronic HCV infected patients compared to healthy donors. Activating receptors are labeled red, inhibitory receptors are labeled light green, and receptors that have both activating and inhibitory functions are labeled yellow, integrins are label blue.

<table>
<thead>
<tr>
<th>IDs</th>
<th>Proteins</th>
<th>Genes</th>
<th>Reported functions</th>
<th>Log2 (BL/H)</th>
<th>BL: level B</th>
<th>Log2 (W56/H)</th>
<th>W56: level B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q10589</td>
<td>Bone marrow stromal antigen 2</td>
<td>BST2 (CD317)</td>
<td>IFN-induced antiviral host restriction factor, moderately restricts HCV production and release from Huh-7.5 hepatocytes\textsuperscript{191}.</td>
<td>-1.16</td>
<td>+</td>
<td>-0.66</td>
<td>+</td>
</tr>
<tr>
<td>E9PNW4</td>
<td>CD59 glycoprotein</td>
<td>CD59</td>
<td>Incorporated into HCV virions and in turn protect HCV from ADCML\textsuperscript{182}. Co-receptor of NKp46 and NKp30 and activates NCRs mediated-cytotoxicity\textsuperscript{177}.</td>
<td>-3.07</td>
<td>+</td>
<td>-2.36</td>
<td>+</td>
</tr>
<tr>
<td>A0A0A0MT22</td>
<td>Protein-tyrosine-phosphatase</td>
<td>PTPRC (CD45)</td>
<td>Protein tyrosine-protein phosphatase required for T-cell activation through the antigen receptor. Regulate DNAM1-mediated killing activity\textsuperscript{192}.</td>
<td>-0.51</td>
<td>+</td>
<td>-0.60</td>
<td>+</td>
</tr>
<tr>
<td>A0A0C4DFY0</td>
<td>P-selectin glycoprotein ligand 1</td>
<td>SELPLG (CD162)</td>
<td>Involved in migration in NK cells. Mediates rapid rolling of leukocytes over</td>
<td>-0.53</td>
<td>-1.48</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Gene ID</td>
<td>Protein Name</td>
<td>Description</td>
<td>p-value 1</td>
<td>p-value 2</td>
<td>Significance</td>
<td></td>
<td></td>
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<tr>
<td>----------</td>
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<td>-----------------------------------------------------------------------------</td>
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<td></td>
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</tr>
<tr>
<td>J3QRA</td>
<td>Fractalkine</td>
<td>CX3CL1 The membrane-bound form promotes adhesion of those leukocytes to</td>
<td>-0.62</td>
<td>-1.05</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>endothelial cells. May play a role in regulating leukocyte adhesion and</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>migration processes at the endothelium</td>
<td></td>
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<tr>
<td>H0YBZ</td>
<td>HLA class II histocompatibility</td>
<td>CD74 A non-polymorphic type II transmembrane glycoprotein. Serves as cell</td>
<td>-0.75</td>
<td>-1.55</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>antigen gamma chain</td>
<td>surface receptor for the cytokine MIF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q15762</td>
<td>Fractalkine</td>
<td>DNAM-1 (CD226) Regulates NK cell antitumor responses</td>
<td>0.73</td>
<td>+</td>
<td>-0.50 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O00481</td>
<td>Butyrophilin subfamily 3</td>
<td>BTN3A1 (CD277) A co-regulator of the immune signal in T and NK cells</td>
<td>4.27</td>
<td>+</td>
<td>5.39 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>member 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q9UGN</td>
<td>CMRF35-like molecule 8</td>
<td>CD300a Contributes to the down-regulation of cytolitic activity in natural</td>
<td>0.45</td>
<td>+</td>
<td>0.59 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>killer (NK) cells 196,197</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Q9Y65</td>
<td>G-protein coupled receptor 56</td>
<td>GPR56 (ADGRG 1) By associating with the tetraspanin CD81 negatively</td>
<td>0.68</td>
<td>+</td>
<td>0.62 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>regulates immediate effector functions, including production of inflammatory</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>cytokines and cytolitic proteins, degranulation, and target cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>killing 198</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0A0G</td>
<td>Leukocyte-associated immunoglobulin-like receptor 1</td>
<td>LAIR1 (CD305) An inhibitory receptor that plays a constitutive negative</td>
<td>1.07</td>
<td>+</td>
<td>1.35 +</td>
<td></td>
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<tr>
<td>2JNK8</td>
<td></td>
<td>regulatory role on cytolitic function of natural killer (NK) cells, B-cells</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>and T-cells 199</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q9Y28</td>
<td>Sialic acid-binding Ig-like lectin 7</td>
<td>SIGLEC7 (CD328) Mediates inhibition of natural killer cells cytotoxicity.</td>
<td>-0.88</td>
<td>+</td>
<td>-0.88 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Expression of Siglec-7 was significantly decreased on NK cells from HCV-infected and HBV-infected patients 200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0A08</td>
<td>CD48 antigen</td>
<td>CD48 As both an activating and inhibitory receptor, either promoting or</td>
<td>-0.90</td>
<td>-1.07</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7X1S7</td>
<td></td>
<td>inhibiting target lysis on NK cells 201</td>
<td></td>
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<td></td>
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<tr>
<td>E5RHN</td>
<td>Hepatitis A virus cellular</td>
<td>HAVCR2 (Tim-3) Regulate NK cells both positively and negatively, depending</td>
<td>-1.11</td>
<td>+</td>
<td>-0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>receptor 2</td>
<td>on the ligand presented by the target cell 202,203</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Q9NQ2</td>
<td>SLAM family member 7</td>
<td>SLAMF7 (CD319) Positively regulates NK cell functions by a mechanism</td>
<td>0.46</td>
<td>+</td>
<td>0.20</td>
<td></td>
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<tr>
<td>5</td>
<td></td>
<td>dependent on phosphorylated SH2D1B. However, in the absence of SH2D1B,</td>
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<td></td>
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<td></td>
<td></td>
<td>inhibits NK cell function 196,204</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Q08722</td>
<td>Leukocyte surface antigen CD47</td>
<td>CD47 Positively and negatively regulates NK cell function, a cell-intrinsic</td>
<td>-0.40</td>
<td>-1.28</td>
<td>+</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>and systemic regulator of NK cell homeostasis and NK cell function in</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>responding to a viral infection 179,217</td>
<td></td>
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</tr>
<tr>
<td>D3DSM</td>
<td>Integrin beta-1</td>
<td>ITGB2 (CD18) Required for optimal NK-cell development and function 206.</td>
<td>0.56</td>
<td>+</td>
<td>0.58 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P26010</td>
<td>Integrin beta-7</td>
<td>ITGB7 An adhesion molecule that mediates lymphocyte migration and homing</td>
<td>0.77</td>
<td>+</td>
<td>0.49 +</td>
<td></td>
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<td></td>
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<td>207.</td>
<td></td>
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<tr>
<td>P20701</td>
<td>Integrin alpha-L</td>
<td>ITGAL (CD11a) Involved in a variety of immune phenomena including leukocyte-</td>
<td>-0.57</td>
<td>+</td>
<td>-0.72 +</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>endothelial cell interaction. Contributes to NK cell cytotoxicity 54.</td>
<td></td>
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</tbody>
</table>
patients after therapy. CD48 and SIGLEC7, receptors that have both activating and inhibitory functions, were persistently down-regulated but the effect on NK cell immunity is not clear. Notably, the expression of several receptors was dysregulated in BL patients but rescued in W56 patients to the level of healthy controls, suggesting the rescued phenotypes of NK cells in these patients. SLAMF7 and HAVCR2, receptors that can both activate and inhibit NK cell immunity, were dysregulated in BL but returned to the healthy level in NK cells of W56 patients.

Interestingly, the activating receptors, Fractalkine (CXCL1), HLA class II histocompatibility antigen gamma chain (CD74) and P-selectin glycoprotein ligand 1 (SELPLG, CD162), were found only dysregulated in W56 patients, supporting the impaired immunity of NK cells after DAA treatment. DNAM-1, an activating receptor in NK cells, was up-regulated in BL but slightly down-regulated in W56 patients, also suggesting impaired immunity in the patients after treatment. Leukocyte surface antigen CD47 (CD47), a receptor can activate antitumor immunity but inhibit antiviral immunity in NK cells 179,217, were down-regulated only in W56 patients, indicating trained immunity against virus infection and impaired immunity against tumor generation.

Taken together, most of the dysregulated activating receptors were down-regulated and all identified inhibitory receptors were up-regulated in the NK cells of HCV patients after treatment, suggesting that NK cell function is impaired in the HCV patients. However, the up-regulation of activating receptors BTN3A1, suggests a highly distinct trained NK cell immunity.

4.1.5.3. Signaling proteins profiles suggest a highly distinct trained NK cell immunity

The signaling molecules play a crucial role in regulating the NK cell immunity via integrating and transducing the signals from activating and inhibitory receptors 59. Interestingly, this proteomic study identified 15 signaling proteins (Supplementary Table S6) that are reported to regulate NK cell responses and 8 of them were dysregulated in the cured patients. The signaling proteins that promote NK cell activation (GRB2, ZAP70, PI3K and SKAP2) were significantly down-regulated and the signaling proteins that inhibit NK cell responses, e.g. PTPN6 (SHP-1) and PEPN11 (SHP-2), were significantly up-regulated in the DAA-treated patients (Figure 18), indicating the impaired NK cell immunity in these cured patients. However, the adhesion and degranulation promoting adaptor protein (ADAP, FYB), which has been shown to be required for cytokine production and degranulation 79,178, was significantly up-regulated in the cured patients, suggesting trained immunity. Furthermore, the β-arrestin 2 (ARRB2), which is essential to inhibit NK cell responses, was found significantly down-regulated in cured patients, further indicating the establishment of the trained immunity in these patients.
Therefore, down-regulation of GRB2, ZAP70, PI3K and SKAP2, as well as up-regulation of PTPN6 (SHP-1) and PTPN11 (SHP-2) indicates the impaired immunity of cured patients. Up-regulation of ADAP and down-regulation of ARRB2 support the trained immunity in these DAA treated patients.

Figure 18 | Expression of signaling proteins. Box and jitter plots show the expression of vesicle transport proteins in healthy controls (H), HCV patients before treatment (BL) and after treatment (W56). Each point indicates the median value of three technical replicates. Box plots show the median and quantiles for each group. The Stars indicate significant differences of protein expression determined by Student’s t-test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4.1.5.4. Altered ER stress and vesicle transport phenotype after treatment

HCV infection has been shown to induce oxidative stress and accumulation of misfolded proteins which is also known as endoplasmic reticulum (ER) stress. The HCV induced ER stress plays important role in developing liver cirrhosis. Besides, ER stress has been reported to reduce the expression of NKG2D on NK cells. Therefore, HCV infection might down-regulate NKG2D in the NK cells of patients by increasing the ER stress. Unfortunately, NKG2D itself was not identified in this proteomic study, however, other proteins (CISD2, CALU and NCK1) that can alleviate ER stress were found persistently downregulated in NK cells both at BL and W56 patients (Figure 19). Insufficiency of CISD2 can disrupt the homeostasis of Ca^{2+} in ER, inducing ER stress and promoting HCC development. The Ca^{2+}-binding protein CALU...
and cytoplasmic protein NCK1 also play important roles in reducing ER stress\textsuperscript{224,225}. Sustained down-regulation of these proteins in the NK cells of BL and W56 patients suggest they had sustained higher ER stress and \textbf{impaired} NK cell immunity, which might contribute to the development of HCC. This is in line with the reports that chronic HCV infection often leads to HCC development\textsuperscript{3} and DAA-cured HCV patients have surprisingly high HCC development and recurrence\textsuperscript{156,157}.

\textbf{Figure 19} | \textbf{Expression pattern of ER stress related proteins.} Box and jitter plots show the expression of ER stress-related proteins in healthy controls (H), HCV patients before treatment (BL) and after treatment (W56). Each point indicates the median value of three technical replicates. Box plots show the median and quartiles for each group. The Stars indicate significant differences of protein expression determined by Student’s t-test: *p < 0.05, **p< 0.01, ***p < 0.001, ****p < 0.0001.

The release of cytotoxic molecules in NK cells requires transport in vesicles, so-called lytic granules\textsuperscript{82}. The budding and fusion of these vesicles with the membrane surface are regulated by cytoplasmic coat protein (COP) complexes: COPI, which buds vesicles from the Golgi apparatus to the ER, COPII, which transport vesicles from the ER to the Golgi apparatus, and clathrin, which regulate the vesicle transport between Golgi, endosomes and surface membrane\textsuperscript{226}. Interestingly, the proteomic data show that the identified components (\textbf{Figure 20}) of COPI (COPA, COPB1, COPB2, COPE), COPII (SEC23A, SEC24C) and clathrin (CTLB and CLTC) were all shifted to even lower expression level in the NK cells of W56 patients, strongly suggest a phenotypic shift instead of “just” slow recovery dynamics. Down-regulation of these vesicle transport machineries indicates that the NK cell degranulation most probably is \textbf{impaired} in cured patients.
Figure 20 | Expression of vesicle transport proteins. Box and jitter plots show the expression of vesicle transport proteins in healthy controls (H), HCV patients before treatment (BL) and after treatment (W56). Each point indicates the median value of three technical replicates. Box plots show the median and quantiles for each group. The Stars indicate significant differences of protein expression determined by Student’s t-test: *p < 0.05, **p< 0.01, ***p < 0.001, ****p < 0.0001.

4.1.5.5. Sustained alteration of RNA processing proteins before and after treatment

RNA processing proteins are not only crucial for the replication of the virus but also important for antiviral responses, because many sensors of the innate immunity can recognize the RNA from virus. Therefore, chronic HCV infection might also affect the RNA processing proteins in NK cells. To test this hypothesis, I have submitted all the identified proteins to DAVID (https://david.ncifcrf.gov/), a web-based tool that can provide functional annotations for proteins and found that 242 proteins were characterized as RNA processing proteins (Supplementary Table S7). In chronic HCV patients, 40 (17%) of these RNA processing proteins were significantly dysregulated, of which only 9 proteins (Table 14) were rescued in the cured patients to the level of healthy individuals while the other 31 proteins displayed sustained dysregulation (Table 15). The direction of altered expression of RNA processing proteins suggests that the antiviral immunity was, depending on the different pathways, either trained or impaired in NK cells of cured patients.
## Results

Table 14 | **Rescued RNA processing proteins in cured HCV patients.** Proteins that were down-regulated in BL but rescued in W56 patient are in green, proteins up-regulated in BL but rescued in W56 patients are in red

<table>
<thead>
<tr>
<th>Protein.IDs</th>
<th>Proteins</th>
<th>Genes</th>
<th>Log2 (BL/H)</th>
<th>Log2 (W56/H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q969Y2</td>
<td>tRNA modification GTPase GTPBP3, mitochondrial</td>
<td>GTPBP3</td>
<td>-2.41</td>
<td>-1.03</td>
</tr>
<tr>
<td>P62273</td>
<td>40S ribosomal protein S29</td>
<td>RPS29</td>
<td>-1.31</td>
<td>-0.71</td>
</tr>
<tr>
<td>P83876</td>
<td>Thioredoxin-like protein 4A</td>
<td>TXNL4A</td>
<td>-2.23</td>
<td>-0.39</td>
</tr>
<tr>
<td>Q9Y4W2</td>
<td>Ribosomal biogenesis protein LAS1L</td>
<td>LAS1L</td>
<td>-1.27</td>
<td>-0.31</td>
</tr>
<tr>
<td>O95218</td>
<td>Zinc finger Ran-binding domain-containing protein 2</td>
<td>ZRANB2</td>
<td>-1.53</td>
<td>-0.11</td>
</tr>
<tr>
<td>Q8WWY3</td>
<td>U4/U6 small nuclear ribonucleoprotein Prp31</td>
<td>PRPF31</td>
<td>0.73</td>
<td>0.41</td>
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<tr>
<td>P62304</td>
<td>Small nuclear ribonucleoprotein E</td>
<td>SNRPE</td>
<td>0.93</td>
<td>0.47</td>
</tr>
<tr>
<td>O15514</td>
<td>DNA-directed RNA polymerase II subunit RPB4</td>
<td>POLR2D</td>
<td>1.06</td>
<td>0.50</td>
</tr>
<tr>
<td>P17252</td>
<td>Protein kinase C alpha type</td>
<td>PRKCA</td>
<td>1.17</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table 15 | **Sustained up- or down-regulated RNA processing proteins in cured HCV patients.** Proteins that were sustained down-regulated are in green, proteins that were sustained up-regulated are in red

<table>
<thead>
<tr>
<th>IDs</th>
<th>Proteins</th>
<th>Genes</th>
<th>Log2 (BL/H)</th>
<th>Log2 (W56/H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O95777</td>
<td>U6 snRNA-associated Sm-like protein LSm8</td>
<td>LSm8</td>
<td>-1.63</td>
<td>-1.69</td>
</tr>
<tr>
<td>P09234</td>
<td>U1 small nuclear ribonucleoprotein C</td>
<td>SNRPc</td>
<td>-1.19</td>
<td>-1.35</td>
</tr>
<tr>
<td>Q5T3Q7</td>
<td>HEAT repeat-containing protein 1</td>
<td>HEATR1</td>
<td>-1.40</td>
<td>-1.20</td>
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<tr>
<td>Q96E39</td>
<td>RNA binding motif protein, X-linked-like-1</td>
<td>RBMXL1</td>
<td>-1.05</td>
<td>-1.01</td>
</tr>
<tr>
<td>P67775</td>
<td>Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform</td>
<td>PPP2CA</td>
<td>-0.76</td>
<td>-0.72</td>
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<tr>
<td>O14744</td>
<td>Protein arginine N-methyltransferase 5</td>
<td>PRMT5</td>
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<td>40S ribosomal protein S8</td>
<td>RPS8</td>
<td>-0.71</td>
<td>-0.65</td>
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<tr>
<td>P0DN76</td>
<td>Splicing factor U2AF 35 kDa subunit</td>
<td>U2AF1</td>
<td>-0.78</td>
<td>-0.62</td>
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4.1.5.6. Enrichment analyses revealed impaired cellular NK cell pathways

To characterize the differentially regulated biological processes in the NK cells of W56 patients in comparison with healthy controls, I classified the proteins into five sections (Figure 21A) and performed systematic pathway enrichment assay using the same method as described before (see Chapter 4.1.4.5). Then the significantly (p<0.05) enriched items were selected and are listed in Supplementary Table S8A (“KEGG pathways” for W56 vs H), supplementary table S8B (“Biological processes” for W56 vs H). Then I selected the items related to NK cell biology and created a heatmap and hierarchical clustering based on the transformed p-values. Expression of components in the chemokine signaling pathway, RNA transport, Jak-STAT signaling pathway, and leukocyte transendothelial migration was enriched in Q1, lower in NK cells from W56 patients than from healthy donors. The expression of components in SNARE interactions in vesicular transport were enriched in Q4, higher in NK cells of W56 patients compared to healthy individuals (Figure 21B). Most of GOBPs related to NK cell immunity, e.g. natural killer cell mediated cytotoxicity, immune effector process, were enriched in Q1 and Q2, lower in W56 NK cells in comparison with healthy donors (Figure 21C).

Figure 21 | Pathway analyses of the NK cell proteome in cured HCV patients. (A) Comparing NK cell proteome of HCV patients after treatment (W56) and healthy donors (H), 5 sections were determined by level A (log2 value of fold change > 0.2, p-value < 0.01) and level B (log2 value of fold change > 0.1, p-value < 0.05) significant regulations in volcano plot: down-regulated in level A (Q1), down-regulated only in level B (Q2), not
Results

significantly regulated (Q3), up-regulated only in level B (Q4), and up-regulated in level A (Q5). These quantiles were evaluated separately for enrichment assay of KEGG pathways (B) and GOBP (C). The proteins were clustered according to the p-values. “+” and “−” are used to indicate the direction of enrichment (“+” for over-representation, “−” for under-representation). The “0” indicates no difference. Significant enrichment (p < 0.05) is visualized in yellow/red for over-represented and in light blue/dark blue for under-represented pathways, whereas non-significant enrichment (p>0.05) is displayed in green.

Comparing the analyses with the conclusion from Chapter 4.1.4.5, the MAPK signaling pathway, proteasome and evasion by virus of host immune response were up-regulated in BL and rescued in W56 patients to the level of healthy controls. In contrast, intracellular receptor-mediated signaling pathways were down-regulated in BL but rescued in W56 patients. However, oxidative phosphorylation, chemokine-mediated pathway, leukocyte transendothelial migration, as well as immunity-related GOBPs, e.g. natural killer cell-mediated cytotoxicity, immune effector processes were sustained impaired in NK cells of both BL and W56 patients. Furthermore, SNARE interactions in vesicular transport was shifted to up-regulated in NK cells of W56 patients, suggesting trained NK cell function. RNA transport, Jak-STAT signaling pathway and Hepatitis C were not significantly regulated in BL but shifted to down-regulated in NK cell of W56, suggesting the impaired immunity of W56 NK cells.

Taken together, these analyses suggest that MAPK signaling pathway, proteasome and intracellular receptor-mediated signaling pathways were rescued, the chemokine signaling pathway, RNA transport, leukocyte transendothelial migration, immune effector process, and natural killer cell mediated cytotoxicity were impaired, and SNARE interactions in vesicular transport was trained in NK cells of cured patients.
4.2. ADAP is required for cytotoxicity and cytokine production in human NK cells

*Adhesion and degranulation-promoting adaptor protein* (ADAP, FYB) was significantly up-regulated (log₂(BL/H)=0.38) in NK cells from chronic HCV-infected patients and stayed up-regulated (log₂(W56/H)=0.45) even after the therapy. In T cells ADAP has been shown to regulate chemokine receptor and T cell receptor (TCR)-mediated integrin activation and promote adhesion and migration²³⁰,²³¹ but its role in NK cells is still elusive. Interestingly, the pathways related to ADAP function, e.g. leukocyte endothelial migration and chemokine signaling pathways, are down-regulated in NK cells from chronic HCV-infected patients (see Figure 21). These data make me wonder what the function of ADAP in human NK cells is, and whether the sustained up-regulation of ADAP belongs to the trained immunity in the cured HCV patients.

4.2.1. ADAP knock-out does not affect proliferation, conjugation and MTOC translocation of human NK cells

Despite the fact that ADAP knock-out mice exist, allowing for various NK functional studies⁶⁶,⁷⁸,⁷⁹, further investigations are still hampered due to the lack of a stable ADAP knock-out cell line. To determine the role of ADAP in the human NK cell function, I generated stable ADAP knock-out (ADAP⁻/⁻) NK92 cell lines utilizing the CRISPR/Cas9 technology. After transfection with the constructed ADAP-sgRNA_PX458 plasmid, around 1% of NK92 cells transiently expressed EGFP protein, sgRNA, and SpCas9 protein. The single GFP⁺ NK92 cells were sorted by flow cytometry into 96-well plates and expanded subsequently. The ADAP knock-out cell line was verified by Western Blot analysis (Figure 22A). Sequencing of the potential mutation site confirmed a 13 bp deletion in the ADAP open reading frame which will generate an unstable mutated peptide (Supplementary Figure S3A&B).

First, I wanted to know whether the proliferation of NK92 cells is affected when ADAP is lacking. I stained the wild type (WT) and knock-out (KO) NK92 cells with CFSE²³² and measured the CFSE intensity every 24 h (Supplementary Figure S3C). The data show that the CFSE signal in KO NK92 cells goes down at a similar rate as in wild type NK 92 cells (Figure 22B&C), which indicates that ADAP is not required for human NK cell proliferation.

In mice NK cells ADAP has been previously shown to be dispensable for conjugate formation with YAC-1 cells⁷⁸. To determine whether ADAP regulates human NK cell conjugation with tumor cells, I incubated PKH26-stained NK92 cells with PKH67-stained K562 cells (E:T=1:1) for 0, 5, 10, 20, 30, 40, 50 and 60 min and measured the percentage of conjugated NK92 cells.
by gating on the PKH67\(^+\) doublets population in PKH26\(^+\) NK92 cells (Supplementary Figure S3D). Consistent with reports for mice, these data indicated that the loss of ADAP does not affect the conjugate formation of NK cells (Figure 22D).

Figure 22 | ADAP knock-out does not affect proliferation, conjugation and MTOC translocation in NK92 cells. (A) Western Blots show the expression of ADAP in WT and KO (ADAP\(^{\text{-/-}}\)) NK92 clones. \(\beta\)-Actin served as the loading control. (B&С) For the determination of the proliferation rate of WT and KO NK92 cells. NK92 cells were stained with 7 µM CFSE for 20 min at room temperature and cultured in 37°C incubator with 5% CO\(_2\) supplemented with 100 U/mL IL-2. The intensity of CFSE was measured continuously for 6 days. Data is one representative of three experiments. (D) Conjugation assay of NK92 cells. NK92 cells stained with PKH26-GL (red) were mixed with K562 cells stained with PKH76-GL (green), incubated at 37°C for 60, 50, 40, 30, 20, 10, 5 and 0 min and then stopped by ice cold 4% PFA. The conjugate rate was calculated based on the ratio of PKH26\(^+/\)/PKH76\(^+/\) doublets in the population of PKH26\(^+\) cells. Data based on three experiments, each has three technical replicates. Error bars represent mean ± SD of three independent experiments (ns: not significant, *: p≤ 0.05). (E) MTOC polarization of NK92 cell. NK92 cells were incubated with CFSE-stained K562 cells for 15 min, stained with Tubulin antibody (YL-1/2) and second antibody (A13c). Distance between the MTOC center and synapse was measured. K562 cells are shown in green and tubulin is shown in red. Scale bar defines 10 µm. (F) Statistics evaluation on MTOC translocation was done to compare WT and KO NK92 cells. Data is one representative of two experiments.

In preparation for the release of lytic granules to kill target cells, the microtubule organizing center (MTOC) is reoriented and polarizes to the immune synapse. In T cells ADAP interacts with dynein thus promoting the translocation of MTOC\(^{233}\). To elucidate whether it is the same
case in NK cells, I monitored the effect of ADAP knock-out for MTOC translocation. I incubated the CFSE-labeled K562 cells and NK92 cells for 15 min at a 1:1 E: T ratio, and then fixed and stained them with a tubulin antibody. Under the microscope, I measured the distance between MTOC and immunological synapse, which is an indicator of polarization (Figure 22E). The statistics (Figure 22F) showed no significant difference in MTOC polarization between WT and KO NK92 cells.

Taken together, these data suggest that the global phenotype in human NK cells, including proliferation, conjugation and MTOC translocation, does not require ADAP.

### 4.2.2. ADAP interacts with SKAP1/SKAP2 in human NK cells

To confirm the specificity of ADAP knock-out and investigate the potential co-regulation of other proteins, I performed iTRAQ-labeled proteomic analyses of the ADAP knock-out and WT NK92 cells. The WT and KO (ADAP−/−) cells were lysed, the proteins were digested with trypsin overnight and differentially labeled with iTRAQ reagent which is a standard used for quantitative mass spectrometry in proteome research. Afterwards, WT and KO samples were mixed at equal amounts, fractionated using basic reverse phase chromatography and measured by LC-MS/MS (Figure 23A). Using Proteome Discoverer searches against the *Homo sapiens* Uniprot database, I identified more than 7000 proteins for each replicate (Supplementary Table S9), with 5885 proteins identified and quantified in all three replicates (Figure 23B). The complete list can be seen in (Supplementary table S9). Consistently, the log2 regulation factors (log2RF) of all three replicates are distributed symmetrically around the median and centralized at the 0 value (Figure 23C), indicating only minimal amounts of up- and down-regulated proteins. For the proteins identified in all three replicates, I created volcano plots (Figure 23D) using the regulation factors (log2(KO/WT)) and P values (−log10P) generated by Student’s t-test. Due to the tendency to underestimate fold changes in iTRAQ, all the regulation factors are “compressed” 235–237. Nevertheless, ADAP has the lowest regulation factor (-2.6), which confirms the knock-out of ADAP. Of note, SKAP1 and SKAP2 proteins, whose stability in T cells is dependent on ADAP 72,238,239 and can regulate T cell activation together with ADAP 240,241, had also markedly lower expression in KO NK92 cells (with regulation factors of -1.8 and -1.6). Therefore, ADAP may also interact with SKAP1 and SKAP2 to regulate NK cell function. However, the other interaction partners of ADAP in T cells, including CARMA1, BLC10, MALT1, Kindlin-3, MST1, RIAM, TAK1, TRAF6, RAP1A, and ZAP70, did not have significantly different expression in WT and KO NK92 cells. The other top candidate proteins found also down-regulated in KO NK92 cells are *twinfilin1* (TWF1), *protein POF1B* (POF1B) and *T cell receptor*...
**Results**

Associated transmembrane adaptor 1 (TRAT1 or TRIM). Both TWF1 and POF1B are actin-binding proteins and potentially regulate cell adhesion\(^\text{242,243}\). TRAT1 is a co-receptor in T cells and it stabilizes the T cell receptor (TCR) through interaction with the zeta chain\(^\text{244}\). On the other hand, granulysin (GNLY), SEMA4A and B-lymphocyte antigen CD20 (MS4A1) had higher expression in the absence of ADAP. GNLY is a component in the cytolytic granule, it induces target cell apoptosis through increasing intracellular calcium and decreasing intracellular potassium\(^\text{188}\). As a cell surface receptor, SEMA4A controls cell migration\(^\text{245}\) and is required for CD8+ T cell activation\(^\text{246}\). MS4A1 is known as a pan B cell marker and involved in signaling downstream of the B cell antigen receptor (BCR)\(^\text{247}\). Higher expression of these proteins indicates that ADAP\(^{-/-}\) NK cells have an even higher capacity to respond to stimulation.

Figure 23 | Workflow and overview of proteomic data. (A) Schematic outline for proteomic analysis. WT and KO (ADAP\(^{-/-}\)) NK92 cells were lysed with lysis buffer, digested with trypsin and labeled with iTRAQ reagent. After measurement by LC-MS/MS, the raw data were processed by Proteome Discoverer to identify proteins and obtain the relative expression level of every protein. (B) Venn diagram indicates the number of identified proteins. In total, 8967 proteins were identified in at least one technical replicate, with 5885 of them present in all three technical replicates. (C) Boxplot showing the distribution of log2 regulation factor (Log2(KO/WT)) for all technical replicates. The thick line in the middle shows the median, boxes indicate the range of regulation factor of 50% of the data and points indicate the outliers in the data. (D) The protein abundances of WT and KO (ADAP\(^{-/-}\)) NK92 cells were compared using Student’s t-test. Based on the p-value (-log10P) and mean of fold change (log2(KO/WT)), the volcano plot was created to show the distribution of regulated proteins. Grey dash lines suggest the thresholds for significant regulation. Proteins that are significantly regulated are shown in red, and proteins that have been shown to interact with ADAP in T cells are shown in black. (E) WT, KO (ADAP\(^{-/-}\)) and rescued (KO-ADAP) NK92 cells were analyzed by Western blotting using the indicated antibodies. One representative experiment of two is shown.
As CRISPR was reported to still have off-target effects \(^{248}\), it is essential to confirm that the altered phenotypes of ADAP\(^{-}\) NK92 cells are caused by the loss of ADAP. Therefore, I cloned the GFP-ADAP sequence into the pBMN-I-GFP plasmid and generated the corresponding retrovirus. Then I transfection this virus into WT and KO NK92 cells, with an empty virus expressing only GFP as control, named as WT-GFP, WT-ADAP, KO-GFP and KO-ADAP clones. The GFP\(^{+}\) NK92 cells were sorted by flow cytometry and expanded afterward. Western Blot data (provided by Dr. Stefanie Kliche) show the expression of ADAP, SKAP1, SKAP2, RAPL and RIAM (Figure 23E) in the expanded clones and also confirm that the SKAP1 and SKAP2 stability is dependent on the presence of ADAP.

To investigate the potential ADAP interaction partners in NK cells, I performed immune-precipitation-coupled mass spectrometry (IP-MS) assays. First I cross-linked the proteins in WT-GFP, WT-ADAP, KO-GFP and KO-ADAP NK92 cells with 0.5% formaldehyde for 10 min, then extracted proteins and immune-precipitated with GFP-Trap resin. The eluates were digested and labeled with iTRAQ 4 \(\times\) plex and then measured by LC-MS/MS (Figure 24A). Afterwards, the intensity of WT-ADAP was divided by WT-GFP to get the ratio in WT background, and the intensity of KO-ADAP was divided by KO-GFP to get the ratio in KO background. The experiments were performed twice thus generating two technical replicates for each condition. Then I calculated the mean of the ratio (\(\log_2(\text{ADAP-GFP/GFP})\)) and P-value from Student’s t-test (-\(\log_{10}\)P) for proteins at least identified in two replicates (Supplementary Table S10) and generated a volcano plot accordingly (Figure 24B). As expected, ADAP shows the highest difference (2.61) which indicates the successful binding of ADAP-GFP to the GFP-trap resin. Due to lower amounts of GFP in WT-ADAP and KO-ADAP than in WT-GFP and KO-GFP clones, GFP has the lowest difference (-2.3). The GFP-enriched proteins, CHD3 and KRT4, are potential contaminants. Notably, SKAP1 and SKAP2 were also co-eluted with ADAP, which demonstrates the robust interaction of ADAP and SKAP1/SKAP2. Interestingly, LYN, a tyrosine kinase protein, was also identified as a potential interaction partner of ADAP although it only showed up in two out of four replicates. LYN has been reported as functionally redundant with FYN in NK cells \(^{249}\), which partly explains the absence of FYN in the data. Besides, the ADAP-interacting proteins in T cells, RAP1A, ZAP70, Kindlin-3, have been found in the IP-MS data. However, their abundances did not have a significant difference in the GFP and GFP-ADAP elutes, suggesting these proteins may not closely interact with ADAP in human NK cells.

Furthermore, I wanted to check whether the interaction between ADAP and SKAP1/SKAP2 is altered in the case of activation. Therefore, I performed co-IP assays on rested or K562 activated...
Results

WT-ADAP or KO-ADAP cells, using rested WT-GFP and KO-GFP cells as control. The eluate was applied to Western blot analysis with antibodies of ADAP and SKAP1/SKAP2. As shown in Figure 24C, both ADAP-GFP and SKAP1/SKAP2 were successfully enriched in WT-ADAP and KO-ADAP cells. K562 stimulation, which initiates NK92 cell degranulation and cytokine production, did not cause a difference in the binding ability of ADAP and SKAP1/SKAP2.

In summary, these data suggest that ADAP may regulate NK cell function through interaction with SKAP1 and SKAP2 proteins. The stability of SKAP1 and SKAP2, similar as in T cells, is dependent on the presence of ADAP.

Figure 24 | Mass spectrometry data identify SKAP1 and SKAP2 as ADAP interaction partners. (A) Schematic workflow for co-immunoprecipitation-coupled mass spectrometry. The proteins in WT or KO NK92 cells over-expressing GFP or GFP-ADAP were cross-linked with 0.5% formaldehyde, lysed, enriched by GFP-trap and then measured with LC-MS/MS. The ratio between GFP-ADAP versus GFP was used to determine the interaction partners of ADAP. (B) The fold change of WT-ADAP/WT-GFP and KO-ADAP/KO-GFP were treated as replicates of ADAP-GFP/GFP and used for Student’s t-test. Based on the p-value (-log10P) and mean of fold change, volcano plot was created to show the distribution of eluted proteins of IP-MS. Proteins that are significantly regulated are shown in red, and proteins that have been shown to interact with ADAP in T cells are shown in black. (C) The eluates of IP-MS were analyzed by Western blotting using the indicated antibodies. One representative of two experiments is shown.
4.2.3. NK cell migration and adhesion require ADAP

Several previous studies clearly indicated that ADAP-deficiency in T cells impairs chemokine-dependent adhesion and migration in vitro and in vivo \cite{69,230,250,251}. To investigate the role of ADAP in NK cell migration and adhesion, WT, KO (ADAP\textsuperscript{−/−}) and KO-ADAP (over-expression of GFP-ADAP in KO) NK-92 cells were used to assess adhesion to the integrin LFA-1 ligand ICAM-1 or the VLA-4 ligand Fibronectin upon stimulation of the chemokine receptor CXCR4 (treatment with corresponding ligand CXCL12). These experiments were performed by Dr. Stefanie Kliche (OvGU, Magdeburg). As depicted in Figure 25A and B, KO NK-92 cells showed a significantly reduced adhesion to ICAM-1 or Fibronectin in response to CXCL12. For both substrates, these adhesive defects were rescued upon re-expression of ADAP to similar levels of WT NK-92 cells. Notably, no differences in surface expression of CXCR4, LFA-1 or CD29 were detectable in these cell lines that might account for differences in adhesion of KO cells (data not shown). MnCl\textsubscript{2} served as the positive control for these adhesion assays because it directly activates integrins by binding to their ectodomain \cite{252}. Furthermore, no differences in adhesion for both substrates between WT, KO and KO-ADAP NK-92 cells were detected in response to MnCl\textsubscript{2} (Figure 25 A&B). Since this stimulus induces integrin activation independently of inside-out signaling events \cite{253}, the data indicate that ADAP is required for CXCR4-induced LFA-1 and VLA-4 activation in human NK cells.

For the migration assay, NK-92 cells were seeded on ICAM-1- or Fibronectin-coated upper wells of the Transwell chambers. On both substrates, CXCL12-induced migration of NK-92 cells was markedly reduced upon loss of ADAP but could be restored by re-expression of ADAP (Figure 25C&D). Migration of T cells along a chemokine gradient requires modifications of the actin cytoskeleton \cite{254,255}. Given that fact that ADAP interacts with Ena/VASP proteins, which modulate the actin cytoskeleton \cite{256}, the F-actin content was determined by intracellular phalloidin staining in these NK-92 cells after CXCR4 stimulation (Figure 25E). In all three cell types there is not any differences in the F-actin content after various time points of CXCL12 triggering. Overall, these data indicate that loss of ADAP in NK-92 cells attenuates CXCR4-induced migration independently of F-actin-driven process.
Figure 25 | ADAP is required for NK cell migration and adhesion. Non-stimulated and CXCL12- or MnCl2-stimulated wild type (WT), KO (ADAP knock-out) and KO-ADAP (over-expression of GFP-ADAP in KO) NK-92 cells were analyzed for their ability to adhere to ICAM-1- (A) or Fibronectin- (B) coated plates. Adherent cells were counted and calculated as a percentage of input (2 × 10^5 cells). Cells were seeded into the ICAM-1- (C) or Fibronectin- (D) coated Transwell inserts. Subsequently, cells were incubated in the absence or presence of CXCL12 in the lower chamber for 2 h. Migrated NK-92 cells in the lower chamber were counted and calculated as the percentage of input (2 × 10^5 cells). (E) WT, KO and GFP-ADAP NK-92 cells were stimulated with CXCL12 (CXCR4 stim.) for the indicated time points, fixed, permeabilized, stained with Phalloidin-Alexa Fluor 633 (F-actin) and analyzed by flow cytometry. Bar plots indicate the mean value of four biological replicates. Error bars represent standard deviation of four independent experiments. Stars indicate significant differences determined by Student’s t-test: *p < 0.05. Experiments were done by Dr. Stefanie Kliche (OvGU, Magdeburg).
4.2.4. ADAP regulates cytokine production and degranulation in human NK cells

To elucidate whether ADAP modulates cytokine production in cellular stimulation in human NK cells, I used K562 (E:T=1:1), or IL-12 and IL-18 plus K562 (E:T=1:1), or PMA plus Iono to stimulate WT, KO (ADAP⁻⁻) as well as the rescued clone KO-ADAP. Monensin and Brefeldin A were added 1 h later to block the transportation of cytokines and IFN-γ expression levels was measured after an additional 5 hours stimulation by flow cytometry (Supplementary Figure S4). When stimulated only with K562 cells the ADAP knock-out NK cells express significantly lower (p < 0.01) IFN-γ than the WT control and this is partially restored when ADAP was rescued in the knock-out clone (Figure 26A). Of note, IL-12 and IL-18 stimulation of NK cell cytokine production was much stronger than tumor cell stimulation and independent of ADAP. Thus, combined stimulation of IL-12, IL-18 and K562 generated a similar amount of IFN-γ in all the NK92 clones (Figure 26B). The capacity to produce cytokines in NK92 cells, indicated by PMA/Iono stimulation, was only minimally affected by the absence of ADAP (Figure 26C). These results suggest that ADAP is involved in the regulation of IFN-γ production in human NK cells.

Figure 26 | ADAP is required for NK92 cell IFN-γ production. WT, KO (ADAP⁻⁻) and KO-ADAP (rescued NK92 cells) were incubated alone (non-stimulated) or stimulated with K562 (A), IL-12 and IL-18 plus K562 (B), or PMA plus Iono (C) for 6 h with the presence of Brefeldin A and Monensin. Then cells were stained with CD56-BV786, IFN-γ-PB and analyzed by flow cytometry to determine the IFN-γ production in terms of MFI and ratio of IFN-γ⁺ cells. The expression of IFN-γ in each clone was normalized by subtracting the value in corresponding unstimulated clones. Data based on three biological replicates, each has three technical replicates. Bar plots indicate the mean value of three biological replicates. Each dot represents the mean of three technical replicates at different
Following polarization, the lytic granules containing perforin and granzymes will be released into the immunological synapse, which is known as degranulation. Since granule polarization and degranulation are controlled by signals from different receptors, I asked the question of whether ADAP plays a role in regulating NK cell degranulation. Therefore, I stimulated WT, KO (ADAP knock-out) and KO-ADAP (rescued NK92 cells) with K562 cells, combined with stimulation of IL-12 + IL-18 and K562, using PMA+Iono stimulation as the positive control (Supplementary Figure S4). The data show that in comparison to WT, ADAP knock-out NK92 cells display significantly (p<0.05) impaired degranulation during stimulation by K562 cells (Figure 27A) or IL-12 + IL-18 + K562 (Figure 27B). In addition, the re-expression of ADAP significantly (p<0.05) enhanced the degranulation response to the WT level under these stimulation conditions (Fig. 27A&B). The capacity for degranulation, which is indicated by PMA/Iono stimulation, is even higher in the KO and KO-ADAP clones (Figure 27C). Together these data show that ADAP is promoting K562 cell-stimulated degranulation in human NK cells.
4.2.5. Cytotoxicity of human NK cells requires ADAP

I am particularly interested in whether the NK cell killing ability is regulated by ADAP. When induced by an NKG2D antibody, in mice the ADAP knock-out has no effect on NK cell cytotoxicity and in human, the ADAP knock-down did not cause significant differences in cytotoxicity in primary NK cells. To clarify the role of ADAP in regulating cytotoxicity, the NK92 cell lines were stained with CFSE and incubated with K562 cells for 4 hours at an E:T ratio of 4:1, and the cells were then stained with 7-AAD to check the death rate of K562 cells (Supplementary Figure S5). As shown in Figure 28, the depletion of ADAP resulted in significantly (p<0.05) impaired cytotoxicity towards K562 cells. Retrovirus transduction-mediated overexpression of GFP or GFP-ADAP in the WT NK92 cells led to only minor differences in killing ability. With only GFP expression, KO cells still have significantly (p<0.01) impaired cytotoxicity, while the GFP-ADAP expression in KO cells enhanced cytotoxicity (p<0.01) back to the WT level. These results show that ADAP is essential for cytotoxicity in human NK cells.

![Figure 28](image)

**Figure 28 | ADAP promotes NK cell cytotoxicity.** NK92 cells were labeled with CFSE, incubated with K562 cells for 4 hours at E: T ratio of 4:1 and then stained with 7-AAD to check the living/dead status of K562 cells and NK92 cells. Data were generated from 3 independent experiments, each has 3 technical replicates. Each dot represents the mean of three technical replicates at different time points. Bar plots indicate the mean value of three biological replicates. Error bars represent the standard deviation of three independent experiments. Stars indicate significant differences determined by Student’s t-test: *p < 0.05, **p < 0.01.

4.2.6. ADAP supports the serial killing activity of NK cells

Cytotoxicity is the outcome of accumulated effects of several rounds of serial killing as each NK cell can only kill one target cell for each contact. Also, only a small subclass of NK cells are responsible for the majority of killing events. Therefore, I asked the question of whether ADAP
Results

promotes cytotoxicity in human NK cells by increasing the proportion of cytotoxic NK cells or through enhancing the target numbers for each NK cell. To analyze serial killing, Dr. Isabel Prager (IfADo, Dortmund) labeled the WT, KO (ADAP knock out) and KO-ADAP (re-expression of GFP-ADAP into KO) NK92 cells with CellTracker and stained K562 cells with SYTOX blue. Then the killing behavior of about 40 individual NK cells for each group was followed over 16 hours in microwells. The results show that almost 80% of WT NK92 cells were cytotoxic and about 60% showed serial killing activity by eliminating up to 6 different targets (Figure 29A&B). Interestingly, for some of the final killing events there was only weak SYTOX blue staining despite a clear apoptotic phenotype of the target, which may be related to a switch in killing mechanism as previously reported 97. In the absence of ADAP, only 40% of the NK92 cells showed cytolytic activity and the serial killing activity was severely impaired with the majority of cells killing only one or two targets (Figure 29C&D). This phenotype was completely rescued by the re-introduction of ADAP in the ko-NK92 cells. To sum up, these data suggest that ADAP controls the serial killing activity of NK92 cells by increasing both the ratio of cytotoxic NK cells and the number of killed target cells for each NK cell.
**Results**

| ADAP controls the serial killing activity of NK92 cells. (A) The indicated NK92 cells were incubated with K562 targets in the presence of the dead cell labeling dye SYTOX Blue using microchips. Cells were tracked over 16 hours via video microscopy. Each contact between an NK92 cell and a K562 cell was characterized in terms of SYTOX Blue staining of the target cell. Each row of the diagram represents one individual NK92 cell, each box displays one contact (serial contacts indicated from left to right) and the color code within box shows killing (blue/light blue) or contacts without killing activity (white). (B) The number of targets killed by each NK92 cell analyzed in (A) is shown as a relative distribution. (C, D) Examples for the killing behavior of individual WT (C) of ADAP knock-out (D) NK92 is shown. NK92 cells (red) are indicated by a yellow asterisk. Killed targets (blue) are numbered in yellow numerals, non-killed targets in white numerals. Arrows indicate the apoptotic bodies of a killed target. Note that the strongly red fluorescent particle in (C) after t=36 min is a dead NK92 cell. Data were collected from two (WT/KO) or three (KO-ADAP) independent experiments. The serial killing experiments were done by Dr. Isabel Prager (IIfADo, Dortmund).
Results
5. Discussion

Many studies have reported that NK cell responses are altered or even impaired in chronic HCV infected patients, but the different HCV genotypes and variant conditions of *ex vivo* assays in these studies did not allow an unbiased general conclusion. In addition, with the successful clearance of HCV by direct-acting antiviral agents (DAAs), many studies have now been conducted to evaluate the NK cell immunity after treatment. However, similar to the situation in chronically infected patients, the phenotype of NK cells after therapy is still under debate, since the heterogeneity of the research backgrounds resulted in inconsistent findings. This project, for the first time, compares the proteomic data of NK cells from chronically HCV infected patients before and after DAA treatment with healthy donors. In total more than 4000 proteins were identified and quantified, covering both surface receptors and the intracellular inventory for NK cell functions, thereby enormously complementing the available data of functional phenotypes of NK cells. The results confirmed the overall inhibited NK cell responses in chronic HCV patients and impaired NK cell immunity in patients after treatment. Based on the collected data, this study investigated the underlying mechanisms for the HCV effect on NK cells and the implications for DAA cured patients. Moreover, this project characterized the critical role of the adaptor protein ADAP in NK cell cytotoxicity via functional assays on wild-type, ADAP knock-out and rescued NK92 cells.

5.1. What are the NK cell phenotypes in chronically HCV-infected patients?

Although some previous reports suggested that chronic HCV-infected patients have a lower ratio of the CD56\(^{\text{dim}}\) NK subset, this observation could not be confirmed in this study: there was no significant subset shift of CD56\(^{\text{dim}}\) and CD56\(^{\text{bright}}\) NK cells in comparison with healthy controls (Figure 11F). The difference may be a result of diverse conditions of detection assays. Since the numbers of CD56\(^{\text{bright}}\) NK cells alone were not sufficient for a proteomic study, both CD56\(^{\text{dim}}\) and CD56\(^{\text{bright}}\) NK cells were collected and used for label-free quantification (LFQ)-based proteomic analyses. To evaluate the proteomic data systematically, I performed both correlation and PCA analyses and found that NK cell proteomes from chronic HCV patients before treatment (baseline, BL) are different from healthy donors and they showed higher inter-individual variation. This is in line with the surface expression analysis from Strunz et al., which showed that the repertoire of NK cell surface markers from chronic HCV patients had much higher donor-to-donor expression variance (DEV) than healthy controls. Next, based on the p-value and fold change of protein abundances, I determined the significantly regulated proteins...
to assess the differential expression profiles between BL patients and healthy donors. This study identified significantly regulated molecules that have not been reported in the context of HCV infection before, e.g. activating receptors (CD59 and CD317), inhibitory receptors (CD300A and LAIR1), antiviral proteins (PRMT5 and HMGN2) and adaptor proteins (ADAP, STAM1 and STAM2). These data thus complement the knowledge of NK cell phenotypes in chronic HCV infection. On the other hand, this proteomic analysis could not identify several receptors (NCRs, TRAIL, NKG2D and NKG2A) that were already reported in chronic HCV infection\textsuperscript{126,135,159}. Overall, the proteomic data of this project suggests that the NK cells in chronic HCV-infected patients expressed a lower level of activating receptors and a higher level of inhibitory receptors, as well as dysregulated antiviral proteins and signaling proteins:

**HCV modulates NK cell activating receptors:** As summarized in Figure 30. Previous reports indicated that the expression of NCRs (NKp30, NKp44 and NKp46), and NKG2D were up-regulated on peripheral NK cells in chronic HCV patients, resulting in enhanced TRAIL-mediated cytotoxicity but dampened IFN-\(\gamma\) expression\textsuperscript{126,135,159}. Other studies reported up-regulation of DNAM-1 and down-regulation of CD16\textsuperscript{258}. The here presented study could not directly detect and characterize the NCRs and NKG2D. However, in line with previous reports, I found that the expression of *DNAX accessory molecule-1* (DNAM-1) was up-regulated in NK cells of chronic HCV patients. As DNAM-1 promotes NK cell cytokine secretion as well as cytotoxicity against tumor cells and virus-infected cells\textsuperscript{259–261}, higher expression of DNAM-1 in the NK cells of BL patients supports activating responses against HCV infection. The expression of CD16, unlike in the previous study, was not significantly altered in the NK cells from chronic HCV patients. This discrepancy may originate from the different methods for measuring CD16: Oliviero et al. used flow cytometry and found that HCV-infected cells down-regulate the expression of surface CD16 on NK cells by metzincin-mediated cleavage\textsuperscript{258}, while I used mass spectrometry and found the total expression of CD16, both in the cytosolic and on the surface, was not altered.

The expression of *butyrophilin subfamily 3 member A1* (BTN3A1, CD277) was up-regulated in the NK cells of BL patients. BTN3A is a member of the B7 family of co-stimulatory receptors that has three isoforms, namely BTN3A1, BTN3A2 and BTN3A3. Compared to the other isoforms, BTN3A2 lacks an intracellular domain and was reported as strongly expressed at the NK cell surface. In contrast, the proteomic data show that BTN3A1 and BTN3A3 represented the main forms in NK cells. This again may originate from different measuring methods. In NK cells, BTN3A1 stimulates NKp30-mediated cytokine production\textsuperscript{195} and up-regulation of BTN3A1 thus suggests also an activating phenotype in NK cells of BL patients. In addition, the
expression of \textit{CD59} glycoprotein (CD59), \textit{bone marrow stromal antigen} 2 (BST-2, CD317), and \textit{receptor-type tyrosine-protein phosphatase C} (PTPRC, CD45) were down-regulated in BL patients in comparison with healthy donors. CD59 has been shown to interact with HCV particles and was identified as a co-receptor of NKP46 and NKP30 in human NK cells and activates NCRs mediated-cytotoxicity against tumor cells. CD317 is an IFN-induced antiviral protein and has the ability to restrict the production and release of HCV particles. CD45 is a tyrosine-protein phosphatase that is necessary for NK cell degranulation and cytokine production against HCMV infection. In summary, there were more down-regulated (CD59, CD317 and CD45) than up-regulated (DNAM-1 and BTN3A1) activating receptors, suggesting inhibited NK cell responses in chronic HCV patients.

**HCV targets NK cell inhibitory receptors:** NK cells from chronic HCV-infected patients have been reported to express a higher amount of inhibitory receptor NKG2A, resulting in reduced cytolytic activity and contributing to NK cell exhaustion. Although NKG2A was not found in this proteomic study, information on other inhibitory receptors could be obtained in this study. \textit{G-protein coupled receptor} 56 (GPR56), an inhibitory receptor expressed on cytotoxic NK and T cells, reduces NK cell effector function against K562 cells. Hitherto the expression of GPR56 in NK cells from chronic HCV patients has not been reported, but in T cells GPR56 was reported to be up-regulated in chronic HCV patients. Similarly, in this project, GPR56 was up-regulated in the NK cells from BL patients. Furthermore, two additional inhibitory receptors, \textit{CMRF35-like molecule} 8 (CD300A) and \textit{leukocyte-associated immunoglobulin-like receptor} 1 (LAIR1), displayed higher expression in NK cells from BL patients than from healthy donors, corroborating the proposed inhibited NK cell cytotoxicity during chronic HCV infection.

**Figure 30 | NK cell adaptation in chronically HCV-infected patients.** Molecular phenotype of NK cells in chronically HCV infected patients as reported by previous studies (A) and as analyzed in the proteome.
study of this thesis (B). Surface receptors are labeled with different colors: activating receptors (red), integrins (blue), inhibitory receptors (green), receptors regulating both activation and inhibitory functions (yellow). The cytoplasmic molecules are clustered in rectangular boxes accordingly: effectors (cyan), antiviral proteins (red) and adaptor molecules (black). The direction of regulation (level B; see chapter 3.7.4) for each molecule was also labeled: up-regulation (↑), down-regulation (↓) and non-significant regulation (≈).

Modulation of other NK cell receptors by HCV infections: this study also identified receptors that can both activate and inhibit NK cell cytotoxicity: CD48, SLAMF7 and hepatitis A virus cellular receptor 2 (HAVCR2, Tim-3). CD48 and its binding partner 2B4 are both expressed on NK cells and target cells. Depending on the cell types and the stage of differentiation, CD48/2B4 interaction can result in activation, co-stimulation or inhibition of NK cell responses. Similarly, the specific function of SLAMF7 also depends on ligands. The potential effects of dysregulated CD48 and SLAMF7 in the NK cells of these BL patients are not clear, and future experiments addressing these proteins could expand our knowledge on these proteins in HCV infections. In NK cells of patients chronically infected with HCV, surface expression of HAVCR2 was reported to be up-regulated and associated with inhibited IFN-γ production and enhanced TRAIL-mediated cytotoxicity. In this project, total HAVCR2 expression levels in the cytosol and at the cell surface were found to be down-regulated in NK cells from BL patients. The inconsistency might originate from the different measuring methods: the previous study measured the surface expression through flow cytometry while this thesis measured the total expression by mass spectrometry. The down-regulation of HAVCR2 suggests the repository of HAVCR2 was exhausted due to sustained HCV stimulation, indicating lower cytotoxicity and higher IFN-γ production. The expression of sialic acid-binding Ig-like lectin 7 (SIGLEC7, CD328) was down-regulated in NK cells of BL patients. SIGLEC7 has been mostly reported to inhibit NK cell cytotoxicity against tumor cells. However, recent studies show that SIGLEC7 can also promote NK cell degranulation and IFN-γ production against K562 tumor cells and lack of SIGLEC7 expression on NK cells is related to dysfunction in chronic HCV infection. Therefore, the down-regulation of SIGLEC7 also most likely indicates that the NK cells in BL patients were inhibited.

HCV modulates integrin signaling: integrins are heterodimeric molecules containing an α and a β subunit that regulate migration and activation of NK cells. However, hitherto the expression of integrins in NK cells in chronic HCV infection has not been reported. This study identified eleven subunits of integrins and three of them were significantly dysregulated in NK cells from BL patients: integrin alpha-L (ITGAL, CD11a) was significantly down-regulated, while integrin beta (ITGB2, CD18) and integrin beta-7 (ITGB7) were significantly up-regulated. Strikingly, CD11a and ITGB2 together constitute LFA-1, which can mediate activation and cytotoxicity through initiating adhesion to the target cell and assembly of the immunological
Down-regulation of CD11a suggests the LFA-1 expression was lower, indicating an inhibited phenotype in NK cells in chronic HCV patients. In addition, ITGB2 can also form macrophage antigen-1 (Mac-1) with integrin alpha-M (ITGAM, CD11b), and form gp150/95 with integrin alpha-X (ITGAX, CD11c). Despite the up-regulation of ITGB2, the expression of integrins Mac-1 and gp150/95 are most probably not affected as both ITGAM and ITGAX (shown in Supplementary Table S4) were not differentially expressed in NK cells from chronically HCV infected patients and healthy individuals. ITGB7 and Integrin alpha-4 (ITGA4) constitute the integrin lymphocyte Peyer patch adhesion molecule (LPAM). Similarly, the up-regulation of ITGB7 does not result in higher LPAM since the ITGA4 was not dysregulated in NK cells of BL patients. To test this, the expression of Mac-1, gp/150/95 and LPAM on NK cells in the chronic HCV patients could be checked by flow cytometry.

**Chronic HCV infection affects the degranulation but not the abundance of lytic granule proteins:** CD107a, a well-accepted marker for NK cell degranulation, was significantly down-regulated in the NK cells from chronic HCV patients, which was not in line with some previous report. The distinct results might be originated from the different measuring methods: previous studies stimulated NK cells with cytokines and measured the surface expression of CD107a, whereas this proteomic study measured total CD107a expression levels in ex vivo isolated NK cells. In contrast, the effector molecules, e.g. perforin, granzymes and granulysin, which are released by the lytic granules were not dysregulated. Thus, NK cells in chronic HCV patients are still “armed”, having the full repository of effector molecules, but potentially cannot degranulate them efficiently towards the target cells.

**HCV infection stimulates MX1/MX2-specific antiviral immunity but inhibits PRMT5- and HMGN2-mediated antiviral responses in NK cells:** Interferon stimulated genes (ISGs), including interferon-induced GTP-binding protein Mx1 (MX1) and interferon-induced GTP-binding protein Mx2 (MX2), were up-regulated in NK cells from chronic HCV patients before treatment (BL). MX1 inhibits HCV replication and promotes other IFNa-mediated anti-HCV activity. MX2 has been reported to interact with NS5A, thereby inhibiting the RNA replication of HCV. Up-regulation of MX1 and MX2 suggests that NK cells in BL patients were responding to HCV infection-stimulated IFNs, although in these cases the ISGs failed to clear the virus. Furthermore, I found that other intracellular antiviral proteins were down-regulated in BL patients, including protein arginine N-methyltransferase 5 (PRMT5) and high mobility group nucleosome-binding domain-containing protein 2 (HMGN2). PRMT5 and HMGN2 are two antiviral proteins that inhibit hepatitis B virus (HBV) replication. PRMT5 interferes with the epigenetic repression of HBV viral DNA transcription and RNA
encapsidation\textsuperscript{279}. HMGN2 inhibits HBV protein expression\textsuperscript{177} and has the capacity to kill tumor cells\textsuperscript{209}. Therefore, down-regulation of PRMT5 and HMGN2 indicates that NK cell antiviral immunity was suppressed by chronic HCV infection, a potential immune evasion strategy for HCV.

**HCV dysregulates signaling proteins in NK cells:** Interestingly, I found that NK cells in BL patients expressed a significantly higher amount of adhesion and degranulation promoting adaptor protein (ADAP) and a lower amount of β-arresting 2. ADAP is required for promoting cytokine production and optimal degranulation in NK cells\textsuperscript{78,79}. β-arrestin 2 has been reported to recruit tyrosine phosphatases and thus facilitating inhibitory signaling in NK cells\textsuperscript{280}. Therefore, the up-regulation of ADAP and down-regulation of β-arrestin 2 support an activated phenotype in NK cells from BL patients in comparison with healthy donors. Interestingly, the tyrosine phosphatase 2 (SHP-2, PTPN11), which is an inhibitory adaptor protein in NK cells\textsuperscript{280}, was found up-regulated in the BL NK cells, supporting inhibited NK cell responses in the chronic HCV patients. Furthermore, two signal-transducing adaptor molecules (STAM), STAM1 and STAM2, had lower expression in NK cells from BL patients than in healthy donors. STAM1 and STAM2 have been reported to participate in the trafficking of cargo proteins to the lysosome for degradation\textsuperscript{211}. STAM1 has been shown to prevent endosomal sorting and degradation of CXCR4 and thereby promoting CXCR4-dependent cell migration\textsuperscript{281,282}. STAM1 and STAM2 are phosphorylated upon cytokine stimulation and are required for the development and survival of T cells\textsuperscript{283}. Likewise, one might expect they are also essential for NK cell development and survival. Taken together, ADAP-dependent activation signaling was up-regulated and β-arrestin 2-dependent inhibiting signaling was down-regulation, suggesting the activated NK cell phenotype in the chronic HCV patients. On the other hand, SHP-2-dependent inhibiting signaling was up-regulated and STAM-dependent activating signaling was down-regulated, indicating the inhibited NK cell phenotype in the chronically HCV-infected patients.

**HCV selectively targets NK cell immune effector pathways:** To evaluate NK cell function of chronic HCV patients at the pathway level, I divided all characterized proteins within this study into five different sections according to their direction and significance of regulation (see chapter 4.1.3). Then these sections were tested for enrichment of functional categories based on annotations of KEGG pathways and gene ontology biological processes (GOBP), using the method adapted from Pan et al.\textsuperscript{167} and Dr. Jenny Voigt\textsuperscript{168}. In line with the molecular phenotypes discussed above, the pathway enrichment assay indeed showed both activating and inhibitory functions in NK cells of chronic HCV patients compared to healthy donors. The activated phenotype includes the enrichment of proteins involved in both MAPK signaling and proteasome
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pathway. MAPK transduces signals for activation of interferon-stimulated genes (ISGs) for antiviral responses \(^{284}\) and proteasome activity is stimulated by the interferons and can also induce the expression of ISGs to promote clearance of virus-infected cells \(^{285}\). During chronic HCV infection, all immune cells are constantly activated by the huge amount of replicating virions and produce a higher amount of inflammation signals \(^{156}\). The up-regulation of these two pathways indicates that NK cells of these BL patients were responding to HCV infection. Other pathways that are specifically related to the innate immune phenotype of NK cells, e.g. chemokine signaling pathway, oxidative phosphorylation, immune effector process, natural killer cell mediated cytotoxicity, were found down-regulated in the NK cells from chronic HCV patients, suggesting that the capacity for antiviral and antitumor responses by NK cells is reduced in these patients as compared to NK cells from healthy individuals.

Generally, the proteomics studies as described in this thesis have complemented our knowledge about the molecular phenotype of NK cell functions and immunity in HCV-infected patients (Figure 33A). The direction of regulation was confirmed for some proteins (upregulated DNM-1 and GPR56; down-regulated SIGLEC7), suggesting that HCV generally dysregulates protein expression, which then becomes apparent both at the total protein amount level as well as at the surface level. Other proteins (e.g. non-regulated CD16 and down-regulated HAVCR2) were found dysregulated in discrepancy with the previous reports. This inconsistency could indicate that HCV infection alters both the expression and transportation of these proteins to the surface, resulting in inconsistent regulation at total protein amount compared to surface levels. Together with the results showing lower expression of Golgi apparatus proteins and vesicle transportation machinery (COPI, COPII and clathrin) (see chapter 4.1.5.4), strongly suggest that in particular molecular vesicle transport is inhibited. Whether this inhibition of vesicle transport is a global adaptation by which HCV promotes its own life cycle in infected cells or whether HCV selectively targets lytic vesicle transport in NK cells to escape from antiviral immune responses remains an open question.

5.2. Are NK cell functions rescued, impaired or trained in cured chronic HCV patients?

All chronically HCV-infected patients analyzed in this thesis project were clear of HCV viral particles after 8 weeks of DAA treatment. Their successful recovery from chronic HCV infections, however, raised a question from the immunological perspective: do the impaired immune responses during chronic HCV infections recover after DAA treatment? To underscore this, NK cells from the same patients one year after therapy were collected and subjected to mass
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spectrometry, and resulting proteomes were comparatively analyzed. The analyses of correlation factors and PCA showed that the cured HCV patients are still different from healthy donors, suggesting either impaired or trained NK cell functions. Actually, a higher number of significantly regulated proteins were found in the NK cells from HCV patients after treatment (W56) than the NK cells from patients before treatment (BL). Furthermore, the higher number of proteins in two regulation profiles (BL≈ W56↓, 359 proteins; BL= W56↑, 614 proteins) indicates a molecular shift instead of “just” a slow recovery. In parallel with the DAA therapy, the heterogeneity of NK cells from patients was decreased to the level of healthy individuals. This is also consistent with the report from Strunz et al., where donor-to-donor expression variation (DEV) in the NK cells of cured patients was normalized to the level of healthy donors. The comparison of molecular phenotypes of NK cells after DAA therapy from previous reports and this study is summarized in Figure 31 and will be discussed in this chapter.

Figure 31 | Alteration of NK cell functions in DAA cured HCV patients. Dysregulated molecular phenotype of NK cells in DAA-cured HCV patients reported from previous studies 159,161,162 (A) and the summary for this project (B). In this project, the surface receptors are labeled with different colors based on their potential role in NK cells: activating receptors (red), integrins (blue), inhibitory receptors (green), receptors regulating both activation and inhibitory functions (yellow). The cytoplasmic molecules are clustered in rectangular boxes accordingly: effector molecules (cyan), antiviral proteins (red) and adaptor molecules (black). The direction of regulation for each molecule was also labeled: up-regulation (↑), down-regulation (↓) and non-significant regulation (≈).

Chronic HCV infection has long-term effects on the abundances of surface receptors: The surface expression levels of NCRs (NKp30, NKp44 and NKp46), NKG2A and NKG2D were reported to be restored in NK cells of the DAA-cured chronic HCV patients 159,161,162. Although this study could not directly detect and characterize the expression of NCRs, NKG2A and NKG2D, this project could complement the expression of many other receptors. The expression of the activating receptor BTN3A1, which stimulates NKp30-mediated cytokine production 195, was sustained up-regulated in W56 patients, implying NK cell responses may be trained for higher cytokine production. Importantly, the sustained down-regulation of activating receptors
(CD59, CD317, CD45 and CD11a) and sustained up-regulation of inhibitory receptors (CD300A, GPR56 and LAIR1) suggest that these receptor-related functions within NK cell from cured HCV-infected patients were impaired. Furthermore, two activating receptors, CD74 and CX3CL1, were shifted from unregulated in patients at BL to down-regulation in NK cells of cured patients at W56. CD74 is a receptor for macrophage migration inhibitory factor (MIF) and activation of CD74 stimulates NF-κB mediated inflammatory responses. CX3CL1 has been shown to promote the adhesion and migration process of leukocytes. Down-regulation of these two receptors suggests that the ability for adhesion and migration, as well as response to MIF stimulation, were impaired in the NK cells from cured patients at W56.

SIGLEC7, CD47, CD48, SLAMF7 and HAVCR2 can either activate or inhibit NK cell immunity based on bound ligands. In chronic HCV infection, SIGLEC7 was reported to promote NK cell immunity. Therefore, the sustained down-regulation of SIGLEC7 supports an impaired NK cell phenotype in patients at W56. CD47 has been reported to regulate NK cell effector protein expression and activation against tumor cells, as well as inhibit the expression of IFN-γ and effector proteins against lymphocytic choriomeningitis virus (LCMV) infection. Therefore, the shift of CD47 to down-regulation in the W56 suggested that the NK cells of cured HCV patients might show impaired immunity against cancer cells but trained immunity against virus infection. The specific roles of CD48 and SLAMF7 in the context of HCV infection are still not clear and therefore cannot generate a clear conclusion. HAVCR2 has been reported to inhibit IFN-γ expression and enhance cytotoxicity in HCV infection. The rescued expression of HAVCR2 in NK cells of W56 patients to the level of healthy donors suggests, in this case, recovered NK cell cytotoxicity and cytokine production.

**Impaired degranulation in cured HCV patients:** The sustained down-regulation of CD107a in NK cells of W56 patients suggests an impaired phenotype, this is not in line with previous reports where CD107a was reported to be recovered to the level of healthy controls. As discussed in chapter 5.1, the contradicting data might be a result of methodological differences where previous studies measured the surface expression of CD107a on stimulated NK cells whereas this thesis measured total CD107a expression in resting NK cells. Expression profiles of perforin and multiple granzymes were similar in the cured HCV patients as in the healthy controls. Hitherto, only a few publications have reported the expression pattern of perforin or granzymes in the NK cells in the case of HCV infection. Consistently, the expression of granzymes B in NK cells of HCV patients was not significantly different from healthy controls. The expression of granzymes B in NK cells of HCV patients was not significantly different from healthy controls.
donors. Taken together, these data suggest that cured patients still have the capacity to kill target cells but the degranulation might be impaired.

MX1/MX2-specific immunity was recovered but PRMT5- and HMGN2-mediated antiviral responses were impaired in NK cells of cure patients: Previous studies reported that DAA treatment could normalize expression of interferon-stimulated genes (ISGs) in PBMCs as a result of decreased levels of IFN in the blood \(^289,290\). Consistently, NK cells of patients at W56 contained similar levels of MX1 and MX2, two important ISGs, as NK cells of healthy individuals. Since these two proteins were up-regulated in the patients at BL, this suggests the recovered IFN-stimulated NK cell functions by DAA treatment and viral clearance. Two other antiviral proteins characterized in this thesis, PRMT5 and HMGN2, remained down-regulated one year after the clearance of HCV, supporting the hypothesis that the PRMT5- and HMGN2-dependent antiviral responses in the NK cells of these cured patients are still impaired.

Signaling protein profiles suggest partly trained and partly impaired NK cell immunity in cured patients: Interestingly, the dysregulated signaling proteins discussed in Chapter 5.1 remained all dysregulated after DAA treatment. The sustained up-regulation of ADAP, which promotes degranulation and cytokine production in NK cells \(^79,178\), and sustained down-regulation of inhibitory adaptor β-arrestin suggest that NK cell immunity was trained in the cured HCV patients. However, two other adaptor proteins STAM1 and STAM2 that might regulate NK cell migration and development stayed down-regulated and the inhibitory adaptor SHP-2 was sustained up-regulated, indicating impaired NK cell phenotypes after DAA treatment. Furthermore, another four signaling proteins (GRB2, ZAP70, PI3K and SKAP2) that positively regulate NK cell immunity were shifted from non-regulation in BL to significantly down-regulation in W56 NK cells, while an inhibitory adaptor, SHP-1, was shifted from non-regulation in BL to up-regulation in the NK cells of W56 patients. Dysregulation of these adaptors in the cured HCV patients suggests their NK cell immunity is impaired. In summary, the up-regulation of ADAP and down-regulation of β-arrestin suggest trained immunity while dysregulation of other adaptor proteins (STAM1, STAM2, SHP-1, SHP-2, GRB2, ZAP70, PI3K and SKAP2) supports impaired immunity in the NK cells of cured patients.

Dysregulated RNA processing proteins indicate long-term effects of chronic HCV infection: As RNA processing proteins have the ability to direct edit or cleave various RNA, they are powerful tools for the host immune system to clear viral RNAs \(^227\). On the other hand, viruses could hijack RNA processing proteins to facilitate their proliferation or disrupt the antiviral responses \(^291,292\). In the proteomic study of this thesis, 242 RNA processing proteins were found and 40 (17%) of them were significantly dysregulated in the chronic HCV patients. After DAA
treatment, only 9 of the dysregulated RNA processing proteins in the NK cells of cured patients were normalized to the level of healthy controls, the other 31 (75%) remained dysregulated (Figure 32), suggesting either trained or impaired NK cell immunity in these cured patients. For example, NK cells could use PRMT5 as powerful antiviral immunity. As described in chapter 5.1, PRMT5 has the ability to inhibit HBV replication through interfering with the encapsidation process. Thus, sustained down-regulation of PRMT5 suggest the NK cell immunity against HBV is impaired in cured patients. On the other hand, HCV might also manipulate some RNA processing proteins, e.g. protein phosphatase 2A (PP2A) that is required for pre-mRNA splicing, to inhibit NK cells immunity. PP2A is a major serine/threonine phosphatase that consists of a catalytic subunit, a structural subunit and a regulatory/variable subunit. Interestingly, PP2A inhibits NK cell cytotoxicity and IFN-γ production and the endogenous inhibitor of PP2A, SET, promotes NK cell cytotoxicity and IFN-γ expression. Moreover PP2A has been shown to interact with HCV NS5A protein and inhibit IFNα-induced antiviral activity against HCV. This proteomic study identified and quantified eleven subunits of PP2A, including one catalytic subunit (PPP2CA), two structural subunits (PPP2R1A and PPP2R1B) and ten regulatory subunits (e.g. PPP2R2A and PPP2R5E). The catalytic subunit PPP2CA and one regulatory subunits PPP2R2A were down-regulated, while another regulatory subunit was up-regulated in the NK cells of cured HCV patients. The regulatory subunit may be responsible for substrate specificity and localization to subcellular compartments and the catalytic subunit has the phosphatase activity. The down-regulation of catalytic subunit PPP2CA might suggest the trained immunity of NK cells in the cured patients. Taken together, the dysregulation of RNA processing proteins suggests both impaired and trained immunity in the NK cells cure HCV patients. Of which, the down-regulation of PRMT5 suggests impaired immunity whereas down-regulation of PP2A indicates trained immunity.

Figure 32 | RNA processing proteins were sustained dysregulated in the cure HCV patients. In total, proteomic study in this thesis identified and quantified 4043 proteins, of which 242 proteins are classified as RNA processing proteins. 40 of these RNA processing proteins were significantly dysregulated in the NK cells of chronically HCV-infected patients compared with healthy controls. After DAA treatment, 27 (75%) of the dysregulated RNA
processing proteins in NK cells from chronic HCV patients were sustained dysregulated in the NK cells from cured patients. Only 9 proteins (25%) were rescued in NK cells of cured patients to the level of healthy controls.

**Altered actin cytoskeletal structures by chronic HCV infection:** As shown in chapter 4.1.5.1 and Table 12, the protein which had the most striking up-regulation in chronic HCV patients before and after DAA treatment was beta-actin-like protein 2 (ACTBL2) (log2(BL/H)=9.44, log2(W56/H)=9.35). Although sharing more than 90% sequence homology with β-actin, ACTBL2 is functionally different from other isoforms of actin (299,300). Increased expression of ACTBL2 has been found in colorectal, pancreatic and hepatoma cancer cells (300–302), suggesting the association of ACTBL2 with tumorigenesis and suppressed immune surveillance. The enormous up-regulation of ACTBL2 in NK cells from chronic HCV patients before and after DAA treatment might indicate an abnormal proliferation of NK cells. But this thesis did not found differences in the absolute numbers and the ratio of CD56dim NK cells (see chapter 5.1 and chapter 4.1.4.1). Knock-down down of ACTBL2 in muscle cells was found to diminish the lateral migration, which might (303), indicating that the ACTN4 might likewise be related to the migration in NK cells. Other components of the cytoskeleton network, including myosin light chain (MYL1) and alpha-actinin-4 (ACTN4), were sustained down-regulated in the NK cells from HCV patients. As the actin cytoskeleton is crucial for transporting organelles and vesicles (304), alteration of these cytoskeletal proteins might suggest abnormal degranulation in the NK cells of HCV patients before and after DAA treatment. Thus, the alteration of these actin-related proteins suggests a major change in NK cell cytoskeletal structures, which might be related to an altered degranulation phenotype in cured HCV patients.

**NK cell immune effector pathways were impaired in the cured patients:** Notably, pathways involved in MAPK signaling, the proteasome, and evasion by virus of host immune response were stimulated at BL (see chapter 4.1.4.5) and recovered in NK cells of patients at W56 (see chapter 4.1.5.6) to the healthy level. On the other hand, pathways involved in oxidative phosphorylation, positive regulation of cytokinesis, detection of external stimulus and intracellular receptor-mediated signaling were less abundant in NK cells of patients at BL but rescued in NK cells of patients at W56 to the healthy level. These normalized pathways suggest that successful clearance of HCV rescued NK cell function. However, pathways that are crucial for NK cell immunity, for instance, the chemokine signaling pathway, leukocyte transendothelial migration, immune effector processes, and natural killer cell mediated cytotoxicity, were affected in NK cells from patients both at BL and at W56. Interestingly, some pathways were found to be down-regulated only in NK cells of patients at W56, for example, RNA transport and T cell receptor signaling pathway. These data strongly suggest that the function of migration and cytotoxicity of NK cells were still impaired in the cured HCV patients.
Taken together, the normalized expression of antiviral proteins MX1 and MX2, as well as HAVCR2, a receptor that inhibits IFN-γ expression and enhances cytotoxicity in the case of HCV infection, indicates recovered NK cell responses in the cured patients. The up-regulation of activating receptor BTN3A1 and the adaptor molecule ADAP, as well as the down-regulation of the inhibitory adaptor protein β-arrestin suggest a trained immunity in the cured HCV patients. However, as shown in Figure 33B, up-regulation of the identified inhibitory receptors, down-regulation of activating receptors, antiviral proteins and adaptor proteins, as well as the impaired migration and cytotoxicity pathway in NK cells suggest the NK cell immune responses in the cured HCV patients were impaired. Because NK cells are crucial for regulating the immune system and killing virus-infected or tumor cells, the overall impairment of NK cell function in the cured patients is an implication that they are still more vulnerable to additional viral infections and the occurrence of cancer. This hypothesis is indeed supported by a number of studies that found similar or even higher rates of HCC development among DAA-treated HCV patients, although this conclusion is still under debate due to a limited number of patients.

5.3. How does HCV alter the NK cell proteome?

The drastic effects of chronic HCV infection on NK cell function raised the question: how does the virus affect the NK cell proteome? Potential answers could be:

**Age effect**: Could the altered NK cell function simply originate from age difference? In this study, the patients are on average ten years older than the healthy individuals (See Table 5).
However, the NK cell compartments are highly stable during adulthood and ten years difference is thus unlikely to contribute to such a remarkable difference in NK cell proteomes. Additionally, the oldest healthy donor is also ten years older than the youngest healthy donor but their NK cell proteomes show much higher correlations coefficients. Thus, it is very unlikely that the altered NK cell function in patients is a consequence of age.

**Subset shift:** Some studies have suggested that chronic HCV infection hampers the development of NK cells, resulting in a shift toward a higher ratio of CD56\(^{\text{bright}}\) and a lower ratio of CD56\(^{\text{dim}}\) NK cells. However, here we observed no obvious difference in the ratios of CD56\(^{\text{dim}}\) NK cells between the patients and healthy donors (See Figure 11F). Importantly, Voigt et al. and Scheiter et al. systemical compared proteomes of CD56\(^{\text{dim}}\) and CD56\(^{\text{bright}}\) NK cells and only identified 76 and 31 significantly dysregulated proteins, respectively. This thesis found much higher numbers of dysregulated proteins (308 in the patients before treatment and 1235 in the patients after DAA treatment) that could not be explained by just a subset shift. Furthermore, the subset specific proteins, e.g. CD56, perforin and granzymes, were not found to be dysregulated in this proteomic study. Therefore, the proteomic difference between HCV patients and healthy controls does not come from the subset shift of CD56\(^{\text{dim}}\) and CD56\(^{\text{bright}}\) NK cells.

**Alteration of cytokine and chemokine signaling pathways:** NK cell responses are regulated by signals from soluble cytokines and chemokines secreted by accessory cells, such as monocytes, dendritic cells (DCs) and macrophages. Chemokines induce NK cell migration to the site of infection while cytokines and other soluble ligands additionally affect transcription and secretion in NK cells. Chronic HCV infection has been shown to up-regulate the expression of soluble cytokines and chemokines in the blood, e.g. IL-18, MIP-1\(\beta\), IL12p40. The alteration of these cytokines can, in turn, shape the responses of NK cells. Proteomic data in this thesis identified the expression of 110 interferon-stimulated genes (ISGs). Out of these 110 identified ISGs (Supplementary Table S11), only 34 of them were dysregulated in HCV patients before treatment and of these, 28 were persistently dysregulated in HCV patients after treatment. These data suggest that the alteration of the NK cell proteomes in chronic HCV
patients is potentially due to affected cytokine and chemokine responses (**Figure 34A**).

**Figure 34 | Mechanism of HCV infection affecting NK cell immunity.** (A) HCV infection induces the expression of cytokines and chemokines (IL-12, IL-18, IFN-α, etc.) in other immune cells, e.g. monocytes, T helper cells, and macrophages. These secreted cytokines in the blood then shape the responses of NK cells. (B) Interaction of HCV-infected cells could activate the metalloproteinase 17 (ADAM-17) in NK cells. The activated ADAM17 then cleave the surface receptors on the NK cells, resulting in dysregulated NK cell immunity. (C) HCV might directly infect NK cells through the entry receptors (e.g. CD81) and other receptors that can be corporate into HCV virions (e.g. CD59). Then the HCV viral protein can affect the immune responses through direct interaction with NK cell proteins e.g. OTUD7B and PP2A.

**Proteolysis of surface receptors:** Many surface receptors are susceptible to cleavage by the membrane-anchored enzyme *a disintegrin and metalloproteinase* (ADAM) \(^{310}\). Proteolysis of surface receptors is, therefore, a potential tool for the virus to manipulate immune system functions. It has been reported that the HCV-induced activation of NK cells results in *metalloproteinase* 17 (ADAM-17)-dependent cleavage of CD16 \(^{258}\). Additionally, chronic HCV patients have decreased expression of a surface receptor, SIGLEC7, on the NK cell surface and increased amounts of soluble SIGLEC7 in the serum, causing lower degranulation and IFN-γ production of their NK cells \(^{200}\). In this project, the expression of CD16 was not altered in NK cells of HCV patients, potentially because the cleaved CD16 at the surface triggers CD16 translation in the cytoplasm, compensating the loss of CD16 at the surface. However, in line with the previous report, SIGLEC7 was persistently down-regulated in HCV patients before and after treatment, most probably contributing to the impaired NK cells function in these patients. Moreover, many other receptors had altered expression levels on NK cells of chronic HCV patients, potentially also mediated by the ADAM-mediated proteolysis (**Figure 34B**). Thus, one could speculate that HCV may manipulate NK cell function by regulating the proteolysis of surface receptors.

**Direct infection of NK cells:** Another possibility is that HCV may directly infect mature NK cells or progenitor cells in the bone marrow. This hypothesis is prompted by the study that the influenza A virus can directly target NK cells and induce apoptosis \(^{311}\). There are already reports
suggesting that HCV was found not only in hepatocytes but also in PBMCs. An in vitro experiments have demonstrated that HCV to be able to infect immune cells, including monocytes, macrophages, B cells and T cells. Until now, however, there is no evidence showing that NK cells can directly be infected by HCV. However, there are several reports investigating the direct or indirect interactions of HCV with NK cell proteins. For example, immobilized and concentrated E2 protein from HCV virions may cross-link CD81, which is the HCV entry receptor, on the NK cell surface and blocks NK cell tyrosine phosphorylation, cytokine production and cytotoxic granule release. In the here presented study, I found several significantly down-regulated proteins, including CD81, CD59, OTUD7B and PP2A, that might directly or indirectly interact with HCV proteins. CD59 has been shown to be incorporated into HCV virions. HCV NS5A protein can (i) interact with OTUD7B and promote its deubiquitinase activity, and (ii) target PP2A for down-regulation of cell growth and survival. Therefore, it is plausible to speculate that HCV might take advantage of these proteins (CD81, CD59, OTUD7B and PP2A) to enter and replicate in NK cells. This hypothesis could be tested by measuring the HCV mRNA level in patients NK cells or by in vitro infections assay on primary NK cells using HCV viral particles.

5.4. How could HCV cause sustained alteration of NK cell function?

Recent studies found that, besides to the adaptive immune system, also the conventional innate immune system can specifically sense and adapt to infections by mounting quantitative and qualitative different responses upon secondary challenge or infection, which was termed “trained immunity”. These memory-like features are generally mediated by epigenetic modifications, similar to the mechanism for developing memory T cells. The memory-like NK cells from HCMV-infected individuals showed enormous long-lasting alterations of DNA methylation and deacetylation in the loci that encode surface receptors and cytokines. In contrast to the 14 days half-life of unstimulated NK cells, these epigenetically modified NK cells can survive for years even in the absence of detectable HCMV. A similar mechanism may also underlie the long-term effects of chronic HCV infection in NK cells. Moreover, the core protein of HCV has been shown to regulate epigenetics in intrahepatic cells and that effect could not be restored by clearance of HCV, suggesting that the epigenetic information of NK cell precursors is also susceptible to HCV-mediated alteration. Thus, the sustained alteration of NK cell function might be a result of HCV induced epigenetic modification on NK cells. Given the fact that HCV is present and replicates in the bone marrow of patients, it is plausible to speculate that the precursors of NK cells are undermined...
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(Figure 35). This hypothesis could be tested by comparing the functional phenotypes of NK cell precursors from chronic HCV patients or cured patients with healthy controls.

Figure 35 | Long-term effects of chronic HCV infection on NK cells. HCV has been reported to infect and replicate in the bone marrow of patients, making it possible to shape the immune responses of NK cell precursors. Similar to HCMV, HCV might also alter the DNA methylation and deacetylation in the genome, resulting in long-term effects on the NK cells, so called memory-like NK cells.

5.5. What is the role of ADAP in human NK cell immune responses?

In the first part of this thesis, the adaptor protein Adhesion and degranulation-promoting adaptor protein (ADAP, FYB) was found sustained up-regulated in HCV patients even after DAA therapy, suggesting trained immunity in NK cells from cured patients (see chapter 5.2). Up to date, most of our understanding of ADAP function has been acquired in T cells, where ADAP has been shown to regulate chemokine receptor and T cell receptor (TCR)-mediated integrin activation and promote adhesion and migration. The specific role of ADAP in NK cells has only been studied in mice and is still elusive. Hitherto, no investigation has been performed in the ADAP function in human NK cells. Therefore, the second part of this thesis focused on whether and how ADAP regulates immune responses in human NK cells.

Firstly, I generated ADAP knock-out NK92 cells and characterized global phenotypes (see chapter 4.2.1). The proliferation rate of ADAP-/- NK cells had no obvious difference with wild-type control cells. This observation is consistent with the finding in murine NK cells by Fostel et al. and different to the report by Srivastava et al. in murine T cells, where ADAP could regulate the cell cycle progression. The ability of NK92 cells to form conjugation with tumor K562 cells was not affected by ADAP knock-out. Again, this is in line with the result of murine NK cells from Fostel et al., but not consistent with findings in T cells of human and mice, where ADAP is required for integrin-dependent T cell conjugate formation. In human T cells, ADAP is crucial for the translocation of microtubule-organizing center (MTOC) to the immunological synapse (IS). But the here presented study revealed that ADAP is dispensable.
for the NK cell MTOC translocation. Taken together, these data suggest that, unlike in T cells, the proliferation, conjugation and MTOC translocation in NK cells do not require ADAP.

Secondly, I analyzed the proteome of ADAP knock-out NK92 cells to investigate the potential co-regulation of other proteins (see chapter 4.2.2 and Figure 23). Overall, the absence of ADAP does not decrease the cytotoxic proteins, e.g. granzymes and perforin. The proteins that were significantly down-regulated in the knock-out clone are SKAP1, SKAP2, POF1, TWF1 and TRAT1. SKAP1 and SKAP2 are the well-known interaction partners of ADAP and their stable expression is dependent on the presence of ADAP in T cells.\textsuperscript{71,72} Down-regulation of SKAP1 and SKAP2 in ADAP knock-out cells was validated by Western-blotting (Figure 23E), suggesting: i) their stability in NK cells is dependent on ADAP as well; ii) they might also constantly bound to ADAP in NK cells. TWF1 and POF1B are actin-binding proteins involved in cell adhesion\textsuperscript{242,243}, and TRAT1 can stabilize T cell receptor (TCR) complexes to facilitate activation of T cells\textsuperscript{244}. Thus, lower expression of TWF1 and POF1B suggests the adhesion ability of NK cells might be affected by ADAP knock-out, and down-regulation of TRAT1 might indicate inhibited NK cell responsiveness in the absence of ADAP, which will be discussed later.

On the other hand, granulysin, SEMA4A and MS4A1 are found to have significantly higher expression in the ADAP\textsuperscript{/}NK92 cells. Granulysin is a cytolytic protein that can kill target cells\textsuperscript{188}. SEMA4A has been shown to be required for cell migration\textsuperscript{245} and activation of CD8+ T cell\textsuperscript{246}. MS4A1 is involved in signaling transduction downstream of the B cell antigen receptor (BCR)\textsuperscript{247}. Therefore, up-regulation of granulysin, SEMA4A and MS4A1 suggest the ADAP knock-out NK cells have an even higher capacity to be activated by target cells. These data suggest that the impaired NK cell cytotoxicity (will be discussed later) was not because of insufficient cytotoxic proteins or activating surface receptors. Other ADAP-interacting partners in T cells, e.g. CARMA1, BLC10, MALT1, Kindlin-3, MST1, RIAM, TAK1, TRAF6, RAPL, and ZAP70\textsuperscript{67}, were also found in the NK cell proteome, indicating the possibility for them to be interaction partners of ADAP. But their expression levels were not regulated in the ADAP knock-out NK cells. Western-blotting also confirmed the stable expression of RAPL and RIAM in NK cells independent from the presence or absence of ADAP (see Figure 23E). Thus, ADAP deletion in human NK cells resulted in the specific co-regulation of SKAP1 and SKAP2 in these cells. The notion that other ADAP-binding proteins were not regulated suggested that other adaptor protein-dependent complexes were available for downstream signaling and effects.

To characterize the interaction partners of ADAP in NK cells, I performed immune-precipitation couple mass spectrometry assays (IP-MS) using the GFP-trap in WT or ADAP knock-out cells over-expressing GFP-ADAP (Figure 24). Only SKAP1 and SKAP2 were found to be solid
interaction partners of ADAP in NK cells and these interactions were not altered by K562 stimulation (Figure 24B&C). Although RAPL, Kindlin-3 and ZAP70 were also identified in the IP-MS data, their expression was not enriched by ADAP, suggesting that they might not interact with ADAP in NK cells. Interestingly, the tyrosine kinase LYN was found to be a potential interaction partner of ADAP, although it was only enriched in one of the two replicates. LYN has been reported to be functionally redundant with FYN, promoting the polarization of cytotoxic granules of NK cells 249. Thus, in NK cells, ADAP might interact with LYN to integrate and transduce activating signals, which still need to be validated by further protein interaction assays. Taken together, these data suggest that ADAP might regulate NK cell responses by interacting with SKAP1 and SKAP2 (Figure 36).

Considering that cell adhesion-related proteins (TWF1 and POF1B), and migration-related protein (SEMA4A) were dysregulated in ADAP knock out NK cells, I speculated that ADAP may also regulate adhesion and migration in NK cells, similar as its role in promoting adhesion and migration in T cells 69,231. Indeed, the data from adhesion and migration assays (see chapter 4.2.3 and Figure 25) showed that upon the stimulation of CXCL12, ADAP is required for optimal adhesion and migration of NK cells to integrin ligands (ICAM-1 and fibronectin). Notably, ADAP is dispensable for the MnCl2-stimulated adhesion and migration of NK cells to integrin ligands. In T cells, CXCL12 stimulation-induced activation of integrin is dependent on the inside-out signaling, while MnCl2 directly activates integrin independently. Therefore, the role of ADAP in regulating adhesion and migration in human NK cells is similar to its role in T cells, depending on the inside-out signaling complex (Figure 36).

Since migration and adhesion are crucial for initiating NK cell responses, one could speculate that following immune responses of NK cells are also affected by the absence of ADAP. For that, I have characterized the role of ADAP in cytokine production and degranulation capacity of human NK cells (see chapter 4.2.4). The data (Figure 26 and Figure 27) showed indeed that ADAP is essential for NK cell IFN-γ production and degranulation upon stimulation by K562 cells, which express ligands for NK cell activating receptors (NKG2D, LFA-1, 2B4 and NCRs). This is in line with a previous report from May et al. 178, which suggested that ADAP promotes degranulation and IFN-γ production in murine NK cells. However, this is not in line with the finding of Rajasekaran, et al. 79, who suggested that ADAP exclusively regulates cytokine production. The inconsistency might due to the different stimulation conditions used in these studies: Rajasekaran, et al. 79 used ligands that can activate either NKG2D or CD137, while this study used intact K562 tumor cells that can activate NK cells via several activating receptors, including LFA-1 328. Although the detailed mechanism for K562-specific activation of NK cells
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is still not clear, one can speculate that LFA-1 is playing an essential role. As it has been shown in this study that ADAP is essential for the LFA-1 mediated adhesion and migration, it is very likely that the tumor cell-dependent stimulation responses of NK cells require ADAP (Figure 36).

As cytotoxicity is the outcome of degranulation and IFN-γ production, one could speculate that the killing ability of NK cells is also regulated by ADAP. Indeed, the cytotoxicity was significantly impaired in ADAP knock-out NK cells and successfully rescued in the NK cells that re-expressed GFP-ADAP (see chapter 4.2.5 and Figure 28). This is contradicting the results from Rajasekaran, et al. 79, which suggested ADAP is not essential for the cytotoxicity of human NK cells against K562 cells. However, the cytotoxicity assay of the previous study used primary human NK cells that had only a marginal knock-down of ADAP. In contrast, this project found that ADAP knock-out in a human NK cell line significantly impaired the cytotoxicity (Figure 36) and a minimal re-expression of GFP-ADAP (Figure 23F) could already rescue these functions. Therefore, the differences in both reports on human NK cell cytotoxicity could be explained by differences in expression levels of ADAP in these cells.

Finally, the cytotoxicity of NK cells occurs in a serial manner and the majority of killing events are performed by only a small subclass of NK cells 94,95. Therefore and building on the above-mentioned phenotypes, it would be of interest to know whether and how the NK cell serial killing is affected by ADAP: either the proportion of NK cells that are responsible for killing or the number of killing events for each NK cell. Thus, the killing behavior of about 40 individual NK cells for each group was followed over 16 hours in microwells. Up to 80% of the wild type NK92 cells were cytotoxic, i.e. able to kill target cells, and 60% of the cells were able to kill even up to 6 different target cells in a row. Intriguingly, only 40% of the ADAP knock-out NK92 cells showed cytotoxic activity and the majority of these cytotoxic NK cells could only kill one or maximally two target cells (Figure 29). Again, these phenotypes were successfully rescued in NK cells that re-expressed ADAP. Taken together, these data suggest ADAP is essential for serial killing by controlling both the ratio of cytotoxic NK cells as well as the number of target cells that could be sequentially killed by each individual NK cell (Figure 36).

In summary, ADAP is essential for the integrin-mediated adhesion and migration in human NK cells and consequently is required for the cytokine production and serial killing by human NK cells (Figure 36).
Figure 36 | ADAP regulates NK cell responses in murine and human NK cells. (A) In murine NK cells, ADAP exclusively regulates cytokine production upon stimulation of CD137 or NKG2D. (B) In human NK cells, stimulation of K562 cells might activate many activating receptors, e.g. LFA-1, NKG2D, NCRs and 2B4. These activating signals transduce through ADAP/SKAP complexes to regulate adhesion and migration, cytokine production, degranulation, and serial killing. ADAP and SKAP1/2 may be involved in LFA-1 mediated adhesion and migration through interacting with complex RIAM/Mst1/Kindlin-3/Rap1 or RAPL/Mst1/Rap1. Additionally, the CBM complex (Card11/Bcl10/Malt1) may also interact with ADAP to promote the activation of human NK cells.

5.6. What are the ADAP interacting partners in NK cells?

Although SLP76 and Carm1 have been suggested as ADAP-interacting proteins in NK cells, no systematic research addressing the interactome of ADAP in NK cells has been performed. In T cells two functionally distinct pools of ADAP signaling complexes exist. One pool of ADAP (~70%) is constitutively associated with SKAP1 and/or SKAP2 and interacts with RIAM/Mst1/Kindlin-3/Rap1 or RAPL/Mst1/Rap1, regulating the inside-out signaling for integrin activation. The other pool of ADAP (30%) is not associated with SKAP proteins but is involved in NK-κB activation through interaction with the Card11/Bcl10/Malt1 complex. Since ADAP is also involved in integrin-mediated adhesion and migration in NK cells, one could envisage that NK cells also have similar complexes for signal transduction. Indeed, all the components of these protein complexes were identified in the proteome of NK92 cells, suggesting that ADAP may also regulate NK cell function by modulating inside-out signaling and NK-κB signaling pathways (Figure 6). Proteomic analysis showed that the stability of SKAP1 and SKAP2 in NK cells depends on the presence of ADAP, whereas all other components were not affected. This may indicate that upon ADAP deletion, NK cell functions
via the SKAP1/SKAP2 pathways may be affected while the CARD11/Bcl10/Malt1 pathway may be unaffected. Further analyses on the downstream events could clarify the function of ADAP in the two separate pathways. Furthermore, IP-MS experiments identified SKAP1 and SKAP2 as ADAP interaction partners, and Western blot analyses on the IP eluates confirmed these interactions (Figure 24C). Taken together, studies in this thesis identified SKAP1 and SKAP2 as ADAP interaction partners in human NK cells (Figure 36) and the validation for other potential interaction partners will be discussed in the outlook (see chapter 7.6).
6. Summary

The function of natural killer (NK) cells is impaired in patients who are chronically infected by the Hepatitis C virus (HCV). The application of direct antiviral agents (DAAs) allows efficient clearance of HCV infection and offers the chance to evaluate NK cell immune responses after treatment. The first part of this thesis aimed to systematically investigate NK cell function in chronically HCV-infected patients before and after DAA therapy, and to answer the two central questions:

6.1. What are the molecular phenotypes of NK cells in chronically HCV-infected patients?

This thesis compared the proteomes of NK cells from chronic HCV infected patients before (BL, baseline) and 56 weeks after the start of DAA treatment (W56) with NK cell proteomes from healthy donors. In total, over 4000 proteins were identified and quantified, and PCA analysis and correlation factor regulations revealed that on the proteomic level NK cells from HCV patients have higher inter-individual variations and are distinct from healthy individuals.

Overall, the proteomic data specified for the first time how NK cell immunity is impaired in HCV infected patients. In total, 308 proteins were determined to be significantly dysregulated. In particular, down-regulation of multiple activating receptors and up-regulation of multiple inhibitory receptors revealed an inhibited phenotype. However, the up-regulation of only a few activating receptors exemplified the more complex phenotypic characterization of NK cells in infected patients. Additionally, degranulation (by CD107a expression levels) but not the cytotoxic molecules were targeted by HCV infection. Higher expression levels of ISGs (MX1, MX2) and the adaptor protein ADAP, as well as the lower expression of inhibitory adaptor β-arrestin, indicated that NK cells were activated by HCV-induced interferons as expected. On the other hand, lower expression of STAM adaptor proteins and antiviral proteins (PRMT5 and HMGN2), as well as higher expression of phosphatase SHP-2 suggested inhibited NK cell migration and antiviral immunity during chronic HCV infection. This is supported by pathway enrichment analysis, revealing that NK cell effector functions (e.g., chemokine signaling pathway, immune effector process, and NK cell mediated cytotoxicity) were down-regulated, and in parallel, the MAPK signaling pathway and the proteasomal pathway were up-regulated. Thus, the NK cell responses were both activated and inhibited by the chronic HCV infection.
6.2. Is NK cell immunity in the cured patients rescued, still impaired or even trained?

The analyses of correlation factors and PCA revealed that NK cell proteomes from cured HCV patients were still different from healthy donors, suggesting long-lasting sustained alteration of NK cell proteomes.

Of the 308 proteins dysregulated in chronically HCV-infected patients, only around 15% were rescued, while about 85% remained dysregulated in NK cells of cured patients. The rescued expression of ISGs (MX1 and MX2) suggested that the NK cell response to interferon stimulation was recovered. However, most of the identified activating receptors were still down-regulated and all identified inhibitory receptors remained up-regulated in NK cells from recovered HCV patients, showing that NK cell immunity was still impaired. Down-regulation of adaptor proteins (STAM1, STAM2) and antiviral proteins (PRMT5 and HMGN2) additionally suggested impaired antiviral immunity in the NK cells of cured patients. Pathway enrichment analysis supported the impaired NK cell function because NK cell effector functions (e.g. chemokine signaling pathway, immune effector process, and NK cell-mediated cytotoxicity pathway) were persistently down-regulated in NK cells from these patients. Interestingly, the upregulation of the activating receptor BTN3A1 and the adaptor protein ADAP, as well as the down-regulation of β-arrestin suggested the presence of trained immunity in the NK cells of cured HCV patients.

Among the proteins that were indicative of impaired or trained NK cell functions, I was particularly interested in the specific role of ADAP. Although the function of ADAP in T cells is well described, the role of ADAP in NK cells is still incompletely understood. In the second part of the thesis, I analyzed the role of ADAP in human NK cells, aiming to answer the following question:

6.3. What is the role of ADAP in human NK cell immune responses?

Newly generated ADAP knock out NK92 cells revealed no obvious global cellular phenotypes: i.e. normal proliferation, conjugation to K562 tumor cells and translocation of the microtubule-organizing center to the immunological synapse. Proteomic comparison wild type and ADAP deficient NK92 cells revealed normal or slightly elevated (granulysin) levels of lytic proteins in ADAP⁻ NK cells. In contrast, the protein levels of SKAP1 and SKAP2 were enormously lower in the ADAP⁺ NK92 cells, suggesting the existence of an ADAP/SKAP1 complex in NK cells. IP-MS experiments indeed revealed, amongst others, that SKAP1 and SKAP2 were genuine ADAP-interacting partners in human NK cells.
NK cell function phenotyping by stimulation with K562 cells revealed that ADAP⁻ NK92 cells produced significantly lower amounts of IFN-γ and showed impaired degranulation efficiency. Furthermore, the killing capacity of ADAP⁻ NK92 cells against K562 cells was remarkably impaired. Interestingly, this study also found that ADAP promotes serial killing by increasing both the percentage of cytotoxic NK cells and the numbers of killed target cells for each single NK cell. Finally, similar to its role in T cells, ADAP was shown to be involved in the regulation of NK cell adhesion and migration when stimulated with CXCL12. Taken together, these data suggest that ADAP is fundamentally required for regulation of human NK cell functions, ultimately resulting in proper cytokine production and full cytotoxicity.

In conclusion, in the second part of this thesis, I discovered that the ADAP/SKAP complex promotes both cytokine production and serial killing through regulating adhesion and migration in human NK cells.

7. Outlook

7.1. Elucidate the function of dysregulated receptors in NK cells in chronically HCV-infected patients

The first part of this study showed that NK cell responses were impaired in chronic HCV patients and that this impairment sustained in the same cured patients even one year after therapy. The direct outcome of impaired NK cell function would be that these cured patients could not effectively clear virus-infected cells upon second infections and/or clear tumor cells. It would, therefore, be of interest to perform ex vivo experiments to compare the killing capacity of NK cells of cured patients against (HCV-) infected cells and tumors. The proteomic study allowed insight into the molecular profiles of activating and inhibitory receptors in NK cells. Multiple receptors (e.g. CD59, CD317, CD45, BTN3A1, CD300A, GPR56, LAIR1 and SLAMF7) have hitherto not been reported in NK cells in the context of chronic HCV infection, it would be of great interest to validate expression patterns in another cohort by using high-throughput flow cytometry. Assuming the regulation profiles of these candidate proteins are validated in a new cohort, a logical next step would be to clarify the function of these receptors in NK cells during chronic HCV infection. This could be addressed by applying corresponding blocking and stimulatory antibodies to primary NK cells, and then measuring their cytotoxic capacity against JFH-Huh7 HCV replication cells. As HCV proteins have been reported to directly interact with surface receptors (CD81 and CD59) on NK cells, it would be also interesting to...
see if any other surface receptors could also interact with viral proteins. One feasible approach would be to incubate fluorescence-coupled HCV virions to NK cells, capture the virion-binding sites using a laser dissection microscope, and characterize these samples using mass spectrometry.

7.2. Characterize the role of dysregulated candidates in NK cell immunity

Antiviral proteins (e.g. PRMT5 and HMGN2) and adaptor proteins (e.g. STAM1 and STAM2, ADAP, β-arrestin) were dysregulated in the NK cells of cured HCV patients. PRMT5 and HMGN2 have been shown to inhibit HBV infection, but their roles in NK cell immunity against HCV infections are still not clear. To explore whether these antiviral proteins contribute to NK cell anti-HCV responses, a plausible experiment would be a knock-down of these proteins in primary NK cells and characterizing their function against JFH-Huh7 HCV replication cells. Similarly, the function of STAM1 and STAM2 has been reported to promote CXCR4-mediated migration in B cells, but its role in NK cell immunity is still elusive. To characterize the specific function of STAM1 and STAM2 in NK cells, firstly, the STAM1 and STAM2 knock out NK cell lines need to be generated and their ability to promote adhesion and migration of these NK cells need to be verified. Secondly, the cytotoxicity of STAM1 and STAM2 knock-out NK cells against HCV-infected cells need to be evaluated to determine whether these adaptor proteins can regulate NK cell effector function. Finally, immune-precipitation can be performed to identify the potential interaction partners of STAM1 and STAM2 in NK cells.

7.3. Identify biomarkers for risk stratification

This study has shown that chronic HCV infection has long-term effects on NK cell immunity, even one year after the DAA therapy. As NK cells are critical for antiviral and antitumor responses, the impaired NK cell immunity in the cured patients implies they are still more vulnerable to virus infections and cancer occurrences. In line with this hypothesis, similar or even higher rates of HCC development in DAA-treated HCV patients have been reported. Therefore, it would be of interest for medical doctors to evaluate the immunity of cured HCV patients and stratify their risk for developing HCC based on biomarkers.

To identify potential biomarkers, the first step would be to determine proteins with distinct expression patterns in NK cells of chronic HCV-infected patients, cured HCV-patients as well as the healthy controls. Therefore, based on the proteins that were significantly dysregulated in the NK cells of patients either before or after DAA treatment, an unsupervised hierarchy clustering heatmap was already constructed (Figure 37). This heatmap clearly showed six major
distinct clusters that could distinguish healthy donors (H), chronic HCV patients (BL) and cured patients (W56). With the collaboration with Prof. Dr. Frank Klawonn, we could already identify 400 proteins as potential biomarkers which can discriminate healthy individuals and chronic HCV patients, 1000 proteins as potential biomarkers to discriminate healthy individual and cured HCV patients, and, most interestingly, 60 proteins that could discriminate patients before treatment and after treatment.

**Figure 37 | Hierarchy clustering heatmap shows the clustering of distinct expression patterns.** Hierarchical clustering of significantly regulated proteins (level A, s0>0.2, FDR<0.01) in BL and W56 patients NK cells. The cluster information of NK cells is shown on the top, while the clustering of specific patterns of regulated proteins is shown on the left. The color scale represents the scaled abundance of each protein, denoted as d 2 (squared Euclidean distance), with red indicating high abundance and blue indicating low abundance.

To validate in particular these 60 candidate biomarkers in a novel cohort in the context of the long-term clinical outcome of cured patients, regarding HCV relapse, HCC development or other diseases caused by impaired immunity. Prominent biomarkers indicative for NK cell immunity, which are associated with the clinical outcome, could be extracted from the current list. Finally, the resulting potential biomarkers need to be validated and examined in larger cohorts.
7.4. Does HCV directly infect NK cells?

In the here presented proteomic data, CD81, the entry receptor for HCV virions was dysregulated. In addition, HCV has been reported to be able to infect immune cells, including monocytes, macrophages, B cells, and T cells. The observation of a variant CD81 expression makes it plausible to propose that HCV might also be able to directly infect NK cells. This hypothesis could be tested by checking whether HCV mRNAs and/or proteins are present in NK cells from chronically HCV-infected patients. An alternative method is to infect an established NK cell line with a Hepatitis C virus strain and determine whether HCV virions have replicated in these cells.

7.5. Determine the dynamics of ADAP complexes and potential immune-regulatory pathways

In the second part of this study, I have characterized the critical role of ADAP for cytotoxicity in human NK cells. Yet it remains to be answered that which receptors require ADAP for signaling transduction. Because ADAP could regulate K562 cell stimulated cytokine production and cytotoxicity, it should be involved in the CXCR4 and, probably, NKG2D, LFA-1, 2B4 and NCRs. To characterize potential interaction partners, the co-localization of overexpressed GFP-ADAP with these receptors could be analyzed by IF microscopy assays. Alternatively, ligand-based stimulation of these receptors followed by an examination of the phosphorylation state of ADAP, e.g. by Western blot or flow cytometry, could reveal whether ADAP is involved in the signaling transduction.

Following the activation of ADAP, also the down-stream interaction partners are of interest. Besides the method of co-immunoprecipitation, it is possible to compare the phospho-proteomes of activated NK cells with or without ADAP. The proteins that exhibit a lower stoichiometry of phosphorylation in activated ADAP−/− NK92 cells are prone to be regulated by ADAP, and the proteins that show a higher phosphorylation state could be part of an alternative pathway for ADAP-mediated activation. Such a phospho-proteomic study will enable us to classify the function of ADAP-interacting proteins based on their phosphorylation.

7.6. Verify the critical role of ADAP in primary NK cells

A limitation of the second part of this thesis is that all the functional assays were performed using a cell line. It is therefore of great interest to study the role of ADAP in primary human NK cells. Interestingly, patients with deleterious mutations of ADAP have been reported. ADAP mutations/absence in these patients is associated with reduced percentages of mature
megakaryocytes in the bone marrow, increased basal expression of P-selectin and PAC-1, as well as small-platelet thrombocytopenia\textsuperscript{333,334}. Since this thesis showed the critical role of ADAP in NK cell effector functions, an examination of the killing ability of NK cells from these patients would be of clinical importance. In collaboration with Dr. Isabel Prager (Leibniz Research Centre for Working Environment and Human Factors at TU Dortmund, Dortmund, Germany), primary NK cells from patients with ADAP mutations and healthy controls could be isolated and should be subjected to serial killing assays (in preparation). This study would deepen our understanding of NK cell immunity and potentially contribute to the NK cell-based immunotherapy.
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114 ls exhibit enhanced responses against

114 toxicity in Chronic Hepatitis C in an

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Bibliography


Appendix

Supplementary Figures

**Supplementary Figure S1 | Gating strategy for the isolation of NK cells used in proteomic analysis.** Lymphocytes were gated based on SSC/FSC flowed by single cell selection using FSC-A/FSC-H. To exclude T and B cells, only CD14/CD19 negative cells were selected and finally the CD56+CD3-CD4- cells were sorted.

**Supplementary Figure S2 | Determine the significantly regulated proteins.** Volcano plots were created based on fold change (difference in log2 values) and p-value from the Student’s t-test. Then the stringent threshold (level A: log2 value of fold change > 0.2, p-value < 0.01) and less stringent threshold (level B: log2 value of fold change > 0.1, p-value < 0.05) was used to determine the significantly regulated proteins.
Supplementary Figure S3 | Genome and theoretical peptides sequence of KO (ADAP<sup>−/−</sup>) NK92 cells and workflow for proliferation and conjugate assay. (A) Partial genome sequence of WT and KO NK92 cells. Signal guide RNA used for CRISPR/Cas9 is underlined and starting codon is colored in green. (B) Partial protein sequence of ADAP in WT and theoretical mutated peptides in KO NK92 cells. The mutated amino acids are in grey. (C) Gating strategy for proliferation assay. Living cells were gated (SSC-A/FSC-A) followed by single cell selection (FSC-H/FSC-A and SSC-H/SSC-A). Then the Mean Fluorescence Intensity (MFI) of CFSE was used for data analysis. (D) Gating strategy for conjugation assay. All living cells were selected (SSC-A/FSC-A) and NK92 cells were gated (PKH26-red) followed by gating conjugated NK92 cells (PKH67-green). The frequencies of conjugated NK92 cells of parent population was used for data analysis. Axis scaling is linear for scattering signals (FSC, SSC) and logarithmic for fluorescence signals. Numbers indicate frequencies in respective gates.
Supplementary Figure S4 | Gating strategy of cytokine production and degranulation assay. Living cells were gated (SSC-A/FSC-A) followed by single cell selection (FSC-H/FSC-A and SSC-H/SSC-A). Then the NK92 cells were selected based on the expression of CD56 (GFP/CD56-BV786). The MFI and ratio of IFN-γ/Pacific Blue were used for data analysis of IFN-γ production in NK92 cells. The MFI and ratio of CD107a/PerCP-cy5.5 were used for data analysis of degranulation in NK92 cells. Axis scaling is linear for scattering signals (FSC, SSC) and logarithmic for fluorescence signals. Numbers indicate frequencies in respective gates.

Supplementary Figure S5 | Gating strategy for cytotoxicity assay. Living cells were gated (SSC-A/FSC-A) followed by single cell selection (FSC-H/FSC-A and SSC-H/SSC-A). Then the K562 cells were selected based on the weaker signal of CFSE and Cytotoxicity was determined by the ratio of 7-AAD+ in K562 cells. Axis scaling is linear for scattering signals (FSC, SSC) and logarithmic for fluorescence signals. Numbers indicate frequencies in respective gates.
Appendix

Supplementary Tables

Supplementary Table S1 | 4867 proteins identified in NK cells of HCV patients and healthy donors comparison. The table lists the Uniprot accession ID, the number of identified peptides, protein names, gene names, and label-free quantification abundances in 54 measurements (18 samples \( \times \) 3 replicates). The column names of 54 measurements are: Code (e.g. HRW) _ group of samples (H, BL or W56) _ number of replicates. For example, HRW_H_1 means the 1st replicates of donor HRW in healthy (H) group. This table is stored in the USB-stick.

Supplementary Table S2 | 4043 proteins with regulatory information. The table lists the Uniprot accession ID, protein names, gene names, P-value and fold change from Students’ t-test comparing BL and Healthy, significantly regulated in BL at level A (P < 0.01, log2(BL/H) >0.2), significantly regulated in BL at level B (P < 0.05, log2(BL/H) >0.1), P-value and fold change from Students’ t-test comparing W56 and Healthy, significantly regulated in W56 at level A (P < 0.01, log2(BL/H) >0.2), significantly regulated in W56 at level B (P < 0.05, log2(BL/H) >0.1), annotation of gene ontology biological processes (GOBP), annotation of KEGG pathways, and abundances in 18 samples (6 \( \times \) healthy, 6 \( \times \) BL, 6 \( \times \) W56). The abundances were the log2 value of median of 3 technical replicates. This table is stored in the USB-stick.

Supplementary Table S3 | Proteins up-regulated in BL but normalized in W56 to the level of healthy controls.

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<th>Log2 (W56/H)</th>
<th>W56: Level A</th>
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<td>Q93052</td>
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<td>Tyrosine-protein phosphatase non-receptor type 1</td>
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**Supplementary Table S4 | Surface proteins identified and quantified.** Surface proteins that have significantly (less stringent threshold, Level B) altered expression in chronic HCV infected patients compared to healthy donors. **Activating receptors** are labeled red, **inhibitory receptors** are labeled light green, and receptors that have both activating and inhibitory functions are labeled yellow, **integrins** are label blue.
<table>
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<th>IDs</th>
<th>Proteins</th>
<th>Genes</th>
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<td>D3DSM0</td>
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<td>P48960</td>
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## Appendix

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**Supplementary Table S5 A&B | Enriched gene ontology terms comparing BL with healthy.** This table lists all terms of the (A) gene ontology categories Biological Process (BP) and (B) KEGG pathways that reached at least 95% statistical significance in one of the sections of BL versus Healthy comparison (see Supplementary Figure S3A). This table is stored in the USB-stick.

**Supplementary Table S6 | Signaling proteins identified in the NK cells from HCV patients and healthy donors.**
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**Supplementary Table S7 | 242 RNA processing proteins identified in the NK cells from HCV patients and healthy donors.** The table lists the Uniprot accession ID, protein names, gene names, P-value and fold change from Students’ t-test comparing BL and Healthy, significantly regulated in BL at level A (P < 0.01, log2(BL/H) >0.2), significantly regulated in BL at level B (P < 0.05, log2(BL/H) >0.1), P-value and fold change from Students’ t-test comparing W56 and Healthy, significantly regulated in W56 at level A (P < 0.01, log2(BL/H) >0.2), significantly regulated in W56 at level B (P < 0.05, log2(BL/H) >0.1), annotation of gene ontology biological processes (GOBP), annotation of KEGG pathways, and abundances in 18 samples (6 × healthy, 6 × BL, 6 × W56). The abundances were the log2 value of median of 3 technical replicates. This table is stored in the USB-stick.

**Supplementary Table S8 A-B | Enriched gene ontology terms comparing W56 patients with healthy donors.** This table lists all terms of the (A) gene ontology categories Biological Process (BP) and (B) KEGG pathways that reached at least 95% statistical significance in one of the sections of BL versus Healthy comparison (see **Supplementary Figure S3B**). This table is stored in the USB-stick.

**Supplementary Table S9 | 8967 proteins identified in the proteomic analysis of ADAP knock-out NK92 cells.** The table lists the Uniprot accession ID, protein names, gene names and log2 value of ratios (ADAP knock-out divided by wild type) obtained from three technical replicates of iTRAQ-based proteomic analyses. This table is stored in the USB-stick.

**Supplementary Table S10 | 1728 proteins identified in the immune-precipitation coupled mass spectrometry assay.** The table lists the Uniprot accession ID, protein names, gene names and log2 value of ratios (GFP-ADAP eluates divided by GFP eluates) obtained from iTRAQ-based IP-MS analyses of WT or ADAP knock-out cells that over-express GFP-ADAP or GFP. This table is stored in the USB-stick.

**Supplementary Table S11 | 110 interferon-stimulated genes identified in the NK cells from HCV patients and healthy donors.** The table lists the Uniprot accession ID, protein names, gene names, P-value and fold change from Students’ t-test comparing BL and Healthy, significantly regulated in BL at level A (P < 0.01, log2(BL/H) >0.2), significantly regulated in BL at level B (P < 0.05, log2(BL/H) >0.1), P-value and fold change from Students’ t-test comparing W56 and Healthy, significantly regulated in W56 at level A (P < 0.01, log2(BL/H) >0.2), significantly regulated in W56 at level B (P < 0.05, log2(BL/H) >0.1), annotation of gene ontology biological processes (GOBP), annotation of KEGG pathways, and abundances in 18 samples (6 × healthy, 6 × BL, 6 × W56). The abundances were the log2 value of median of 3 technical replicates. This table is stored in the USB-stick.
Supplementary Files

Supplementary File S1 | R code #1

```r
### This is code for making boxplot and jitter plot to visualize protein expression in HCV project
### Code created by Wenjie

### Load packages
library(ggplot2)
library(reshape2)
library(ggpubr)
library(stringr)

# Load proteomic data file
HawaiiPlot_0_as_low_value <- read.excel()
LFQ.median <- as.data.frame(HawaiiPlot_0_as_low_value[,c(11:13,21:39)])

# The starting column for protein abundances
s_column <- 5

# Group names for all samples
name_groups <- gl(3,6,labels=c("H","BL","W56"))

## Define function for making plot
# Two inputs needs: "Names" to define protein or gene names, "column" to define in which column the names can be find
medianMSplot <- function(Names, column)
{
    # Extract the row contains proteomic data of "Names" at corresponding "column", complete match
tempdf <- LFQ.median[,grep(paste('^',Names,"$",sep=""), LFQ.median[,column])]
    # Create dataframe suitable for ggplot2
tempdf.gg <- melt(tempdf, measure.vars = colnames(tempdf)[s_column:ncol(tempdf)],
                      variable.name = "Sample", value.name = "abundance")
    
    # Assign group labels to respective samples, based on groupSize
tempdf.gg$Group <- name_groups

    ## Create boxplot with errorbar
tempdf.gg$Group <- name_groups
    medianMSplot <- ggboxplot(tempdf.gg, x="Group", y="abundance",width=0.5, size=0.5, outlier.shape=NA,
                                bxp.errorbar=T, bxp.errorbar.width = 0.4)+
    # Add jitter plot on top
    geom_jitter(aes(color = Sample, shape=Group), size = 1.5, width = 0.05)+
    
    ## Colors for each sample
    scale_colour_manual(values=c("#6C3483","#E74C3C","#3498DB","#27AE60","#85929E","#F1C40F","#FF0000","#E000FF","#FBFF00","#00FF00","#00FFF0","#0000FF"))

    # Alternative way to color all samples
    #scale_colour_manual(values=rc("blue",6).rc("red",6).rc("green",6))+
    # Add p values to to plot
    stat_compare_means(method = "t.test",
                       tip.length = rep(0.03,6),
                       size=2,
                       label = "p.signif",
                       comparisons = list(c("H","BL"), c("BL","W56"), c("H","W56")))+
}
```

132
# Make columns for the color legend
guides(color= guide_legend(ncol = 3))

# Make columns for the shape legend
guides(shape= guide_legend(ncol=3))

# Title of plot--gene name
labs(title = tempdf.gg[1,3],
     subtitle = str_wrap(tempdf.gg[1,2],width = 30, indent = 0),size=4)

# Don't show name for x axis
xlab(NULL)

# Name for y axis
ylab(expression(paste("Log" ["2"],"(LFQ abundance)")))

# Change background
theme_classic(base_size = 10)

## Parameter for plot
theme(legend.title = element_text(size = 8),
      legend.text = element_text(size = 7),
      plot.subtitle = element_text(hjust = 0.5, size = 8),
      plot.title = element_text(hjust = 0.5),
      legend.position = "none")

return(medianMSplot)

# Example for making a plot
medianPlot <- medianMSplot("PML",3)

# Save the plot
print(medianPlot)
ggsave("GNLY-.png",medianPlot.width = 80, height = 80,units = "mm")

---

### Supplementary File S2 | R code #2

#### R code #2: Determination of over- and under-represented GO categories ####

The core of this code was kindly developed and provided by Prof. Frank Klawonn.

Addition of the correction for multiple testing based on Benjamini-Hochberg.

Adapt: Directly to export the newly generated table with raw p and corrected p values (line 96).

Adapt: Directory to initially load table with GO categories assigned to proteins (line 98).

1. install.packages("XLConnect", repos = "http://cran.case.edu")
2. library("XLConnect")
3. get.terms <- function(x,sep=";")
4. all.str <- c()
5. for (i in 1:length(x))
6. if (!is.na(x[i]))
7. all.str <- c(all.str,separate.by(x[i],sep=sep))
8. return(levels(factor(all.str)))
Appendix

23
24
25
26 separate.by <- function(s, sep=";", "){
27 g <- gregexpr(";", s)
28 if (g[[1]][1] == -1)
29 return(s)
30 } else{
31 sep.pos <- c(0, g[[1]], nchar(s) + 1)
32 no.terms <- length(sep.pos) - 1
33 strs <- rep("", no.terms)
34 for (i in 1:no.terms)
35 strs[i] <- substr(s, sep.pos[i] + 1, sep.pos[i + 1] - 1)
36 }
37 return(strs)
38 }
39
40
41 fisher.quantile.term <- function(quant, ft.quants, fts)
42 {
43 inds <- subset(1:length(quants), quant == quant)
44 n.q <- length(grep(ft, fts[inds]))
45 n.other <- length(grep(ft, fts[-inds]))
46 return(fisher.test(matrix(c(n.q, length(inds) - n.q, n.ner, length(quant) - length(inds) - n.ner), nrow = 2), alternative = "less")$p.value)
47 }
48
49
50 fisher.sign.quantile.term <- function(quant, ft.quants, fts)
51 {
52 inds <- subset(1:length(quants), quant == quant)
53 n.q <- length(grep(ft, fts[inds]))
54 n.other <- length(grep(ft, fts[-inds]))
55 pvl <- fisher.test(matrix(c(n.q, length(inds) - n.q, n.ner, length(quant) - length(inds) - n.ner), nrow = 2), alternative = "less")$p.value
56 pvg <- fisher.test(matrix(c(n.q, length(inds) - n.q, n.ner, length(quant) - length(inds) - n.ner), nrow = 2), alternative = "greater")$p.value
57 return(pvg < pvl)
58 }
59
60
61
62
63
64 compute.ft.p.values <- function(quant, fts, ft.set)
65 p.value <- rep(1:length(ft.set))
66 for (i in 1:length(ft.set))
67 p.value[i] <- fisher.quantile.term(quant, ft.set[i], quants, fts)
68 }
69 return(p.value)
70 }
71
72
73
74 compute.ft.sign.p.values <- function(quant, fts, ft.set)
75 p.sign <- rep(1:length(ft.set))
76 for (i in 1:length(ft.set))
77 p.sign[i] <- fisher.sign.quantile.term(quant, ft.set[i], quants, fts)
bonfholm.correction <- function(p.values) {
  ord <- order(p.values)
  psort <- sort(p.values, index.return=T)
  nonas <- sum(is.na(p.values))
  p.values.bh <- rep(1,length(p.values))
  previous.p.value <- 0
  for (i in 1:nonas) {
    p.values.bh[ord[i]] <- p.values[ord[i]]*(nonas+1-i)
  }
  if (p.values.bh[ord[i]]>1) p.values.bh[ord[i]] <- 1
  if (p.values.bh[ord[i]]<previous.p.value) p.values.bh[ord[i]] <- previous.p.value
  previous.p.value <- p.values.bh[ord[i]]
  if (BL_diff BL_B BL_A)

  for (i in 1:nrow)

  BL_A <- rf[i,1]
  BL_B <- rf[i,2]
  BL_diff <- rf[i,4]

  if (BL_A == "+") {
    if (BL_diff>=0) Q_info[i,1] <- "Q5"
    else Q_info[i,1] <- "Q1"
  } else if (BL_B == "+") {
    if (BL_diff>=0) Q_info[i,1] <- "Q4"
    else Q_info[i,1] <- "Q2"
  } else Q_info[i,1] <- "Q3"

  Q_info
  dat.BL <- cbind(Q_info, rf[,c(8,4,7)])
  names(dat.BL) <- c("quantile","accession","log2RF","GOBP","GOCC","KEGG")

  for (i in 1:nrow)

  rf <- HawaiiPlot_0_as_low_value[,c(5,8,18,20,21,11:13)]
  rf$W56_A[is.na(rf$W56_A)] <- 0
  rf$W56_B[is.na(rf$W56_B)] <- 0
  Q_info <- matrix(nrow=nrow,ncol=1)
  for (i in 1:nrow)
  BL_A <- rf[i,1]
  BL_B <- rf[i,2]
  BL_diff <- rf[i,4]
Supplementary File S3 | R code #3

### R code #3: log10(10) transformation and heatmap generation

1. install.packages("grid", repos = "http://cran.case.edu")
2. install.packages("RColorBrewer", repos = "http://cran.case.edu")
3. install.packages("scales", repos = "http://cran.case.edu")
4. if (BL_A == "+"){
   if (BL_diff > 0) { Q_info[i,1] <- "Q5" }
   else { Q_info[i,1] <- "Q4" }
   else if (BL_B == "+"){
      if (BL_diff > 0) { Q_info[i,1] <- "Q4" }
      else { Q_info[i,1] <- "Q2" }
   }
   else { Q_info[i,1] <- "Q3" }
}

### p value correction for multiple testing is based on the Benjamini-Hochberg method

1. p.val.mat <- matrix(1, nrow=length(fits), ncol=length(quantile.names)) # p-value matrix: matrix type declaration (?); number of columns is three times the number of quantile names (3x 5 quantiles)
2. sign.mat <- matrix(1, nrow=length(fits), ncol=length(quantile.names)) # matrix with the regulation direction information: number of columns = number of quantiles (5 columns).
3. for (j in 1:length(quantile.names)){
   p.val.mat[,3*j-2] <- compute.ft.p.values(toString(quantile.names[j]), dat[,1], dat[,3+i], fits)
   p.val.mat[,3*j-1] <- p.adjust(p.val.mat[,3*j-2], method="BH") ### p value correction for multiple testing is based on the Bonferroni-Holm method
   p.val.mat[,3*j] <- bonfholm.correction(p.val.mat[,3*j-2]) ### p value correction for multiple testing is based on the Bonferroni-Holm method
}
4. for (k in 1:length(quantile.names)){
   pvn[3*k-2] <- paste("p.value.raw.", quantile.names[k], sep="")
   pvn[3*k-1] <- paste("p.value.BenjHoch.cor.", quantile.names[k], sep="")
   pvn[3*k] <- paste("p.value.BonfHolm.cor.", quantile.names[k], sep="")
   sn[k] <- paste("is.higher.", quantile.names[k], sep="")
}
5. colnames(resmat) <- c(onto.names[i], pvn, sn)
6. write.csv2(resmat, file = paste(dirp, onto.names[i], ",", csv", sep=""))
7. col.num <- seq(2,14, by=3)
install.packages("graphics", repos = "http://cran.case.edu")
install.packages("grDevices", repos = "http://cran.case.edu")
install.packages("table", repos = "http://cran.case.edu")
install.packages("stats", repos = "http://cran.case.edu")
install.packages("pheatmap", repos = "http://cran.case.edu")
library("grid")
library("RCColorBrewer")
library("scales")
library("graphics")
library("grDevices")
library("stats")
library("pheatmap")

# Part 1: -log10 transformation of corrected p values and adjusting of prefix to the direction of regulation (over- or under- representation of GO term) ###

#Loading of file with GO terms significantly enriched in at least one of the quantiles; based on Benjamini-Hochberg corrected p values #
t_W56vH_GOBP <- read.csv2("U:/Lab work/HCV Project/2018-10-02 GO-based functional analysis from Jenny/R files/W56vH_All 3 pvalues_GOBP.csv", stringsAsFactors=F, header=T)
t_BLvH_GOBP <- read.csv2("U:/Lab work/HCV Project/2018-10-02 GO-based functional analysis from Jenny/R files/BLvH_All 3 pvalues_GOBP.csv", stringsAsFactors=F, header=T)
t_W56vH_KEGG <- read.csv2("U:/Lab work/HCV Project/2018-10-02 GO-based functional analysis from Jenny/R files/W56vH_All 3 pvalues_KEGG.csv", stringsAsFactors=F, header=T)
t_BLvH_KEGG <- read.csv2("U:/Lab work/HCV Project/2018-10-02 GO-based functional analysis from Jenny/R files/BLvH_All 3 pvalues_KEGG.csv", stringsAsFactors=F, header=T)

col.num <- seq(3,15,by=3)
P_transform <- function(t.Name_file)
{
  t <- t[rowSums(t[,col.num] <= 0.05) >= 1,]
c # -log10 transformation of the corrected p-values of selected GO terms #
t_log.p.Q1 <- -log10(t[,2])
t_log.p.Q2 <- -log10(t[,3])
t_log.p.Q3 <- -log10(t[,4])
t_log.p.Q4 <- -log10(t[,5])
t_log.p.Q5 <- -log10(t[,6])

# Adjusting of prefix sign of -log10(corr p-values) to the direction of regulation. Down-regulated GO terms ("False") are made negative. Up-regulated GO terms ("TRUE") remain positive.#
for (i in 1:length(t_log.p.Q1))
  if (t[is.higher,Q1[i]] == "FALSE") t[p.with.dir,Q1[i] <- t[p.with.dir,Q1[i]]*(-1) #pwd = p value with direction
else if (t[is.higher,Q1[i]] == "TRUE") t[p.with.dir,Q1[i] <- t[p.with.dir,Q1[i]]
for (i in 1:length(t_log.p.Q2))
  if (t[is.higher,Q2[i]] == "FALSE") t[p.with.dir,Q2[i] <- t[p.with.dir,Q2[i]]*(-1)
else if (t[is.higher,Q2[i]] == "TRUE") t[p.with.dir,Q2[i] <- t[p.with.dir,Q2[i]]
for (i in 1:length(t_log.p.Q3))
  if (t[is.higher,Q3[i]] == "FALSE") t[p.with.dir,Q3[i] <- t[p.with.dir,Q3[i]]*(-1)
else if (t[is.higher,Q3[i]] == "TRUE") t[p.with.dir,Q3[i] <- t[p.with.dir,Q3[i]]
for (i in 1:length(t_log.p.Q4))
  if (t[is.higher,Q4[i]] == "FALSE") t[p.with.dir,Q4[i] <- t[p.with.dir,Q4[i]]*(-1)
else if (t[is.higher,Q4[i]] == "TRUE") t[p.with.dir,Q4[i] <- t[p.with.dir,Q4[i]]
for (i in 1:length(t_log.p.Q5))
  if (t[is.higher,Q5[i]] == "FALSE") t[p.with.dir,Q5[i] <- t[p.with.dir,Q5[i]]*(-1)
else if (t[is.higher,Q5[i]] == "TRUE") t[p.with.dir,Q5[i] <- t[p.with.dir,Q5[i]]

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# Creation of signs to be displayed in the heatmap: ‘+’ for over-representation, ‘–’ for under-representation, ‘0’ for p=1 (no regulation) #

t.dir.sign.Q1 <- ifelse(t$p.with.dir.Q1>0,"+", ifelse(t$p.with.dir.Q1<0,"–","0"))
t.dir.sign.Q2 <- ifelse(t$p.with.dir.Q2>0,"+", ifelse(t$p.with.dir.Q2<0,"–","0"))
t.dir.sign.Q3 <- ifelse(t$p.with.dir.Q3>0,"+", ifelse(t$p.with.dir.Q3<0,"–","0"))
t.dir.sign.Q4 <- ifelse(t$p.with.dir.Q4>0,"+", ifelse(t$p.with.dir.Q4<0,"–","0"))
t.dir.sign.Q5 <- ifelse(t$p.with.dir.Q5>0,"+", ifelse(t$p.with.dir.Q5<0,"–","0"))
rownames(t)<-1:nrow(t)
Name_save <- paste("U:\Lab work\HCV Project/2018-10-02 GO-based functional analysis from Jenny\Name_file_.csv",sep = "")
write.csv2(t, file=Name_save, row.names=FALSE)
return(t)

### Part 2: Visualization of GO categories and p value matrix in a hierarchically clustered heatmap ###

# row.num <- c(1,3,7,8,14,15,16,17,21,24,25,28,34,36,38,39,42,44,45) #W56 V H KEGG
row.num_W56vH_GOBP <- c(1,3,7,8,14,15,16,17,21,24,25,28,34,36,38,39,42,44,45) #W56 v H KEGG
row.num_W56vH_GOBP <- c(1,3,7,8,14,15,16,17,21,24,25,28,34,36,38,39,42,44,45) #W56 v H KEGG
row.num_BLvH_GOBP <- c(114,130,170,219,230,302,301,320,438,441,454,461,562,564) #BL v H GOBP
#t.BLvH <- t[ row.num,1:2]
row.num <- unique(grep(paste(t.BLvH_GOBP,row.num.BLvH_GOBP,1), collapse="|",sep=""))
Heat_pdf <- function(t,row.num.Name_file, pdf_height)
{
t <- t[row.num,]
p <- data.matrix(t[17:21]) #use of -log10 p-values corrected for the direction for heatmap, i.e. the values are negative or positive depending on the direction of regulation
row.names(p) <- t[1,]

# Definition of breaks for the colour breaks: -log10(0.05) = 1.3010299566398 #
b1 <- c(seq(min(p),-1.3010299566398,by=0.01)) # intervall[lowest p value ; -0.05](- -> -log10 transformed), increment steps: 0.01
b2 <- c(seq(-1.3010299566397,0, by=0.01)) # intervall[-0.05 ; 0](- -> -log10 transformed), increment steps: 0.01
b3 <- c(seq(0.00000001, 1.3010299566397, by=0.01)) # intervall[0 ; +0.05](+ -> -log10 transformed), increment steps: 0.01
b4 <- c(seq(1.3010299566398, max(p), by=0.01)) # intervall[+0.05 ; highest p value](+ -> -log10 transformed), increment steps: 0.01
bk <- c(bk1,bk2,bk3,bk4)

# Definition of colours for the defined intervals #
col1 <- c(colorRampPalette(colors = c("blue","cadetblue1"))(n = length(bk1)-1))
col2 <- c(colorRampPalette(colors = c("green","darkgreen"))(n = length(bk2)-1))
col3 <- c(colorRampPalette(colors = c("darkgreen","green"))(n = length(bk3)-1))
col4 <- c(colorRampPalette(colors = c("yellow","red"))(n = length(bk4)-1))
cols <- c(col1, col2, col3, col4)

#Legend:
legendbreak = c(-4, -1.30103,0.1,30103,3, 6, 10, 20, 30,33) #values which are labeled in the legend (-log10 transformed p values) -> adjust to the minimum and maximum of the data set
legendlabel = c(0.0001,0.05,1.0,0.05,0.001,10^-6,10^-10,10^-20,10^-30,10^-33) #legend labels: corrected p values (not transformed)
pagesToOutputOn = 8  # factor with which A4 length is multiplied; extension of A4 page through multiplication is needed in case of a high number of GO terms
### Heatmap as pdf file which shows “+/-0” to indicate the direction of regulation (Up- or down-regulation of GO term) ###
Name_save <- paste("U:\Lab work\HCV Project/2018-10-02 GO-based functional analysis from Jenny\".Name_file, \_pdf\_sep = ")
pheatmap(p. color=cols. border_color = "grey60". cellwidth = 10. cellheight =9. fontsize =7. 
clustering_distance_rows = "euclidean". clustering_method = "average". 
# Additional display of signs (+/-0) to visualize the direction of regulation of the respective GO term
main = "GOBP pathway")  # Title of the heatmap
# Text boxes (grid.text) are placed by providing the x,y-coordinates (adjustment by trial-and-error)
gtextp("Patients": x=0.375. y=0.597. just=c("centre", "centre"). rot=0. check.overlap=F. name=NULL.
gp=gpar(fontsize=8. fontface="bold"). draw=TRUE. vp=NULL)
gtextp("Healthy": x=0.457. y=0.597. just=c("centre", "centre"). rot=0. check.overlap=F. name=NULL.
gp=gpar(fontsize=8. fontface="bold"). draw=TRUE. vp=NULL)
gtextp("enriched": x=0.572. y=0.597. just=c("centre", "centre"). rot=0. check.overlap=F. name=NULL.
gp=gpar(fontsize=8. fontface="bold"). draw=TRUE. vp=NULL)
gtextp("not enriched \& (p-value): x=0.72. y=0.394. just=c("centre", "centre"). rot=0. check.overlap=F. name=NULL.
gp=gpar(fontsize=8. fontface="bold"). draw=TRUE. vp=NULL)
dev.off()
}

Heat_pdf(t_BLvH KE GG.row.num_BLvH KE GG."BLvH KE GG",2)
Heat_pdf(t_W56vH KE GG.row.num_W56vH KE GG."W56vH KE GG",2)
Heat_pdf(t_BLvH GOBP.row.num_BLvH GOBP."BLvH GOBP",2)
Heat_pdf(t_W56vH GOBP.row.num_W56vH GOBP."W56vH GOBP",2)
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