Analysis of NK cells in intravenous drug users exposed to Hepatitis C

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Contents

1 Introduction

1.1 The Hepatitis C Virus (HCV) infection

About 150 million people worldwide are chronically HCV infected and 3-4 million people are newly infected every year (WHO 2013). After an incubation period of 6 to 8 weeks mild liver inflammation occurs, however, 75% of infections remain asymptomatic. Approximately 80% of infected individuals proceed to develop chronic HCV infection, characterized by HCV specific antibodies and viral ribonucleic acid (RNA) in their blood. 10% to 20% of chronically HCV infected individuals develop liver cirrhosis and about 4% of these develop hepatocellular carcinoma, making HCV the major cause of liver transplantations in western industrial countries (Brown 2005). About 20% of infected individuals spontaneously resolve HCV infection. Viral RNA becomes undetectable in the blood of these individuals, while HCV specific antibodies remain detectable and serve as a marker for previous HCV infection.

Intervention strategies to prevent HCV transmissions in high-risk groups showed only limited success and prophylactic vaccination against HCV is not available to date. Therefore, antiviral therapy is currently the only means to fight HCV. Sustained viral response and clearance of HCV-RNA from patients with chronic HCV infection can be achieved by therapy with pegylated interferon-α (IFN-α) in combination with Ribavirin. However, overall success rates are about 50% and strongly vary between HCV genotypes. Novel drugs directly acting against HCV (DAAs) have been recently approved for therapy and will further enhance the sustained response rates. However, the enormous costs of these novel treatment options will preclude their widespread use in countries with limited public health resources.

1.2 The HCV virion

The Hepatitis C Virus is a positive-stranded RNA virus and belongs to the family of Flaviviridae together with yellow fever virus and dengue virus being other prominent members of this family. Together with the recently identified non-primate, rodent and bat hepaciviruses (NPHV (Kapoor et al. 2011), RHV (Kapoor et al. 2013) and BHV(Quan et al. 2013)) they are grouped into the *Hepacivirus* genus.

Infectious enveloped HCV virions are 50 to 80 nm in diameter. E1 E2 glycoprotein heterodimers are embedded in the lipid bilayer surrounding the nalyser din , consisting of core proteins and the RNA genome (Gastaminza et al. 2010) (Figure 1.1). HCV virions, existing as lipo-viro-particles (LVPs), are not icosahedral, and because of their association with low-density and very-low-density lipoproteins (LDL and VLDL) in the infected host (Andre et al. 2002; Merz et al. 2011), they are pleomorphic with heterogeneous and low density, which varies depending on growth conditions (Lindenbach et al. 2006).
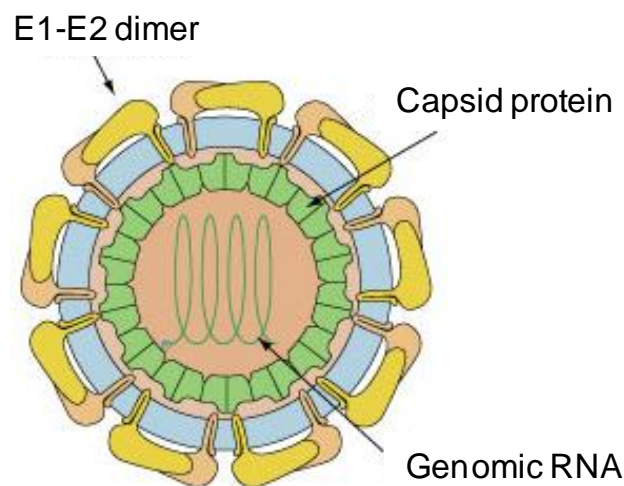
Figure 1.1: HCV virion.
Schematic drawing of an HCV virion. (Modified from http://viralzone.expasy.org).

1.3   HCV genome and genome organization

HCV contains a single-stranded, positive-sense RNA molecule, 9.6 kilo bases (kb) in length coding for one open reading frame (ORF) comprising approximately 3000 amino acids (aa) (Bartenschlager et al. 1993). Translation produces one large polyprotein which is cleaved by host and viral proteases into ten viral proteins: core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Unlike Flavi- and Pestiviruses, HCV RNA has no cap-structure at its 5' end (Figure 1.2). The ORF is flanked by the 5' and 3' untranslated regions (UTRs). The 5'-UTR is a well-conserved 341 nucleotide sequence element that precedes the internal ribosome entry site (IRES) mediating translation of the polyprotein (Tsukiyama-Kohara et al. 1992). The

approximately 200 nucleotides long 3'-UTR is located downstream of the coding region. It can be divided into 3 parts (Hsu et al. 2003): The stop codon of the ORF is followed by a variable region (nalyse. 40 nucleotides), a poly-uracil track of 20 to 90 nucleotides and a highly conserved sequence of 98 nucleotides, which is essential for replication (Kolykhalov et al. 1996; Yanagi et al. 1999).
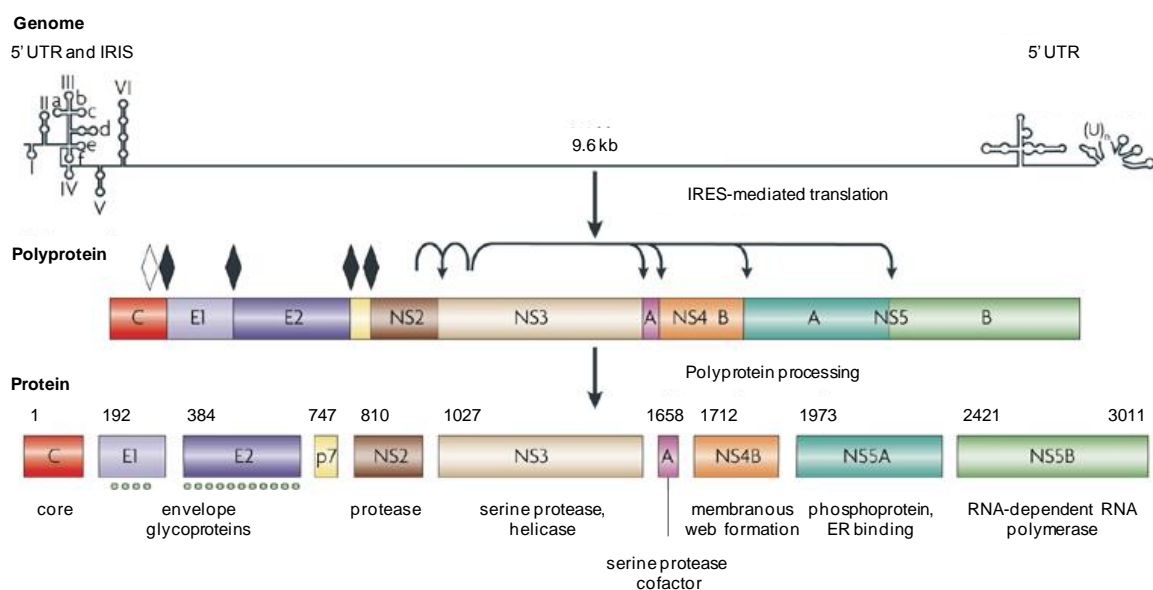
Figure 1.2: HCV genome, polyprotein and proteins.
The 9.6 kb positive-strand RNA genome is schematically depicted at the top. IRES-mediated translation yields a polyprotein precursor that is processed into the mature structural and non-structural proteins. Amino acid numbers are shown above each protein. Solid diamonds denote cleavage sites of the HCV polyprotein precursor by the endoplasmic reticulum (ER) signal peptidase. The open diamond indicates further C-terminal processing of the core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2–3 and NS3–4A proteases. Dots in E1 and E2 indicate the glycosylation of the envelope (Modified from (Moradpour et al. 2007)).

1.4 The HCV virus life cycle

The HCV life cycle is a complex process involving many cellular and host proteins, recently reviewed by Scheel et al. (Scheel et al. 2013). An overview over viral entry and uncoating, translation and polyprotein processing, HCV RNA replication as well as virus assembly and release is given in the following.

1.4.1 Entry and uncoating

A growing number of host proteins have been identified to be involved in successful entry of HCV into hepatocytes. Initial low affinity cell binding is thought be mediated by the LDL receptor and glycosaminoglycans (Agnello et al. 1999; Barth et al. 2003),

before E1-E2 interacts with the co-receptors scavenger receptor class B type I (SR-BI) and CD81 (Pileri et al. 1998; Scarselli et al. 2002). Claudin-1 (CLDN1) and Occludin (OCLN) are proteins typically found in tight junctions and are also required for HCV entry (Evans et al. 2007; Ploss et al. 2009), possibly causing HCV virions to translocate to tight junctions (Figure 1.4). Epidermal growth factor receptor (EGFR) and ephrin receptor type A2 are believed to further modulate CD81 and CLDN1 interaction (Lupberger et al. 2011). Uptake occurs via clathrin-mediated endocytosis (Blanchard et al. 2006). E2 is believed to interact with CD81 (Sharma et al. 2011) and was predicted to be a class II fusion protein activated by low pH in the endosome. These processes lead to the release of the viral genome into the cytoplasm where primary translation can occur (Figure 1.3 (a), (b)). Direct cell-to-cell transmission probably also occurs in the liver.

1.4.2 Translation and polyprotein processing

The HCV genome is highly structured with essential RNA regulatory elements in the 5' and 3' UTRs as well as in the coding region (Gottwein et al. 2008). The IRES located in the HCV 5' UTR initiates Endoplasmatic reticulum (ER)-associated translation (Hoffman et al. 2011),resulting in a polyprotein that is co- and post-transcriptionally cleaved by cellular proteases (signalase and signal peptide peptidase) and the viral NS2-NS3 and NS3-NS4A protease to release the 10 HCV proteins (Figure 1.3 I).

1.4.3 HCV RNA replication

NS4B and NS5A induce the formation of ER-derived membrane spherules. In aggregate those are referred to as the membranous web where RNA replication is believed to take place (Egger et al. 2002; Romero-Brey et al. 2012). The NS5A phosphoprotein consists of three subunits. NS5A domains I and II are essential for RNA replication (Tellinghuisen et al. 2004; Tellinghuisen et al. 2008) while domain III assists in viral assembly (Appel et al. 2008; Tellinghuisen et al. 2008). NS5B is the RNA-dependent RNA polymerase. The NS3 protein is capable of nucleic-acid binding and 3' to 5' translocation coupled to hydrolysis of ATP (Raney et al. 2010). Although its exact role is unknown it might contribute to separation of nascent and template

RNA strands, unwinding RNA secondary structures or displacement of RNA-binding proteins (Figure 1.3 (d)).
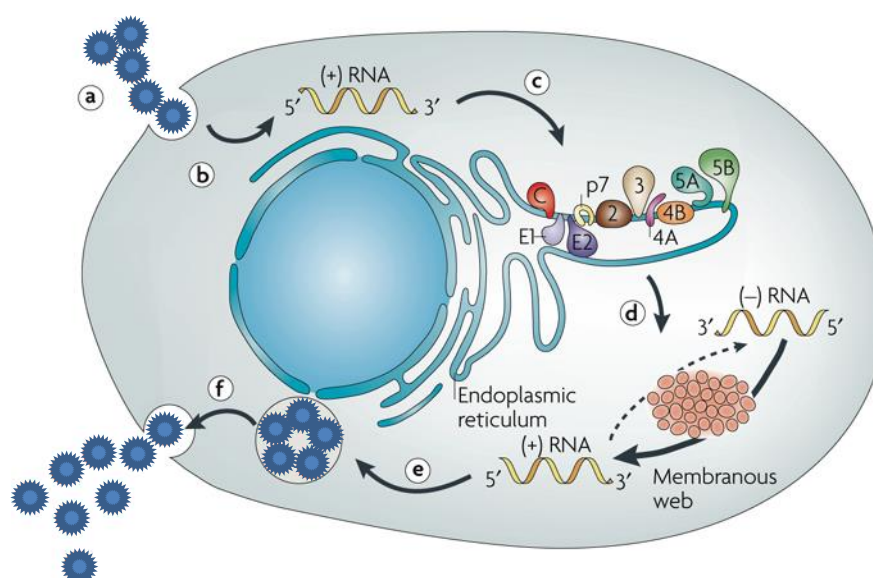
Figure 1.3: HCV life cycle.
Schematic depiction of (a)Virus binding and internalization (b); cytoplasmic release and uncoating (c); IRES-mediated translation and polyprotein processing (d); RNA replication (e); packaging and assembly (f); virion maturation and release . The topology of HCV structural and non-structural proteins at the endoplasmic reticulum membrane is shown schematically (Modified from(Moradpour et al. 2007)).

1.4.4 Virus assembly and release

Virus assembly and release is a tightly regulated process coupled to host cell lipid synthesis (reviewed in (Scheel et al. 2013)). After cleavage by host cell peptidases the mature core protein relocates from ER membranes to cytoplasmic lipid droplets (cLDs) (McLauchlan et al. 2002; Boulant et al. 2006). The current model for nalyser din formation involves interaction of core with NS5A, either on cLDs, whereto NS5A is directed by diacylglycerol acyltransferase-1 (DGAT-1), or after translocation from the mobile cLDs to the ER (Miyanari et al. 2007; Shavinskaya et al. 2007; Appel et al. 2008). Delivery of the HCV RNA genome is probably facilitated by the close proximity of sites of RNA replication and virion assembly and by NS2-coordinated virion assembly through interactions with the glycoproteins, p7, NS3 and NS5A. A series of signal and stop-transfer sequences orchestrate ER translocation of the E1 and E2 glycoproteins, which assume a type I membrane protein topology.

After folding, heterodimer formation and addition of L-linked sugars, the E1-E2 glycanes are then trimmed by glycosidases I and II (Lavie et al. 2007) (Figure 1.3 (e), (f)).

1.5 HCV genotypes and distribution

Due to the missing proof-reading function of the HCV RNA-dependent RNA polymerase there is a high error rate in genome replication resulting in approximately 10^4 to 10^5 nucleotide exchanges per generation (Holland et al. 1982; Drake et al. 1998). Frequent point mutations result in a swarm of heterogeneous but closely related virus variants in the same host termed quasispecies. The balance between mutation and selection within certain populations is believed to result in different viral genotypes (Domingo et al. 2006). Based on genetic divergence HCV can be subdivided into 7 genotypes (1-7) with approximately 30% sequence divergence and multiple subtypes (a,b,c…) with up to 20% sequence divergence (Gottwein et al. 2008). Distinct genotypes predominate in different parts of the world. Genotype 1 dominates in North America (70%), Japan (75%) and Europe (50 to 70%), genotypes 2 and 3 are also frequent in these regions. Genotypes 3 and 6 are widespread in South Asia and South East Asia and genotype 4 and 5 are most common in Africa, but have recently also spread to Europe. Genotype 7 was recently found in a few patients from Central Africa.

Disease progression is largely similar across genotypes. For reasons yet unknown different genotypes show different response rates to treatment with IFN-based therapies. A sustained viral response (SVR) after treatment is obtained in nearly 80% of genotype 2 and 3 infected individuals and only 50% of genotype 1 infected individuals (Manns et al. 2006). Inhibitors of the non-structural proteins NS3-NS4A protease proofed to be a powerful antiviral drug for HCV treatment. However, owing to genotype-dependent efficacy, NS3-NS4 protease inhibitors Telaprevir and Boceprevir are approved for treatment of genotype 1 only. The results achieved with this treatment led to SVR rates as high as 75% (Jensen 2011).

1.6 HCV epidemiology

HCV was assigned to the family of NonA-/NonB hepatitis viruses for a long time before Michael Houghton and his group managed to isolate viral RNA from an infected chimpanzee and created cDNA clones in 1989 (Choo et al. 1989). In fact 80% to 90% of NonA-/NonB hepatitis could be attributed to HCV (Alter et al. 1989).

It has been suggested that the initial spread of the virus started during the last century through the use of unsafe parenteral injections, invasive medical and surgical procedures and transfusion of blood products and was subsequently spread by intravenous drug use among younger people. HCV prevalence in European counties is heterogeneous ranging from less than 0.5% prevalence in Scandinavian countries to up to 7% to 20% in Italy and Greece (Figure 1.4) (Dellabona et al. 1994; Stroffolini et al. 1995; Guadagnino et al. 1997; Lionis et al. 1997; Osella et al. 1997; Goritsas et al. 2000; Maio et al. 2000; Raffaele et al. 2001), The high HCV prevalence in these areas is owed to the widespread use of unsafe medical procedures in the distant past (Wasley et al. 2000; Shepard et al. 2005; Prati 2006). In the late 1980s, 2% to 10% of blood units in developed countries were HCV infected, thus successfully spreading the infection (Alter et al. 1981; Colombo et al. 1987; Esteban et al. 1990; Prati 2006). Nowadays the risk to receive an HCV positive blood transfusion is 1:100000 and no case of HCV transmission due to the administration of plasma-derived products has been reported since 1994 (Prati 2006) leaving intravenous drug use as the major cause of HCV transmission.

1.6.1 Injection drug use and the HCV epidemic

As reviewed by Esteban et al. (Esteban et al. 2008) intravenous drug use has become the main transmission mechanism for HCV in Western Europe (Shepard et al. 2005), accounting for 60% to 90% of prevailing infections in certain European countries (Harris et al. 1999; Westin et al. 1999; Dalgard et al. 2003; Hutchinson et al. 2006; Sutton et al. 2006). Estimates by the European Monitoring center for Drugs and Drug Addiction (EMCDDA) and Eurosurvaillance suggest a rate of 5.3 intravenous drug users (IDUs) per 1000 population aged 16 to 64, resulting in at least 1.7 million IDUs in European countries alone.

Figure 1.4: Estimated HCV prevalence in different European countries.
(Esteban et al. 2008)

1.6.2 Injection drug use and the HCV epidemic

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HCV transmission is reported to occur rapidly after initiation of intravenous drug use especially within the first year (Sutton et al. 2006). After 5 years 50% to 90% of users have been exposed to HCV (Villano et al. 1997). Additional factors associated with an increased risk of HCV infection include age, duration and frequency of IDU, sharing equipment, polydrug use, HCV prevalence among experienced IDUs,

homelessness and having served a prison sentence (Villano et al. 1997; Garfein et al. 1998; Hagan et al. 2001; Judd et al. 2005; Mathei et al. 2006; Hickman et al. 2007). Most new HCV infections occur in young IDUs and the proportion of patients with IDU-related chronic HCV has surpassed those infected by other routes (Haushofer et al. 2001; Bourliere et al. 2002; Schroter et al. 2002; Gerard et al. 2005; Payan et al. 2005; Katsoulidou et al. 2006). In addition, IDU-related genotypes (1a, 3a and 4) have replaced the classical transfusion associated genotypes 1b and 2 among blood donors and young patients (Haushofer et al. 2001; Bourliere et al. 2002; Schroter et al. 2002; Gerard et al. 2005; Payan et al. 2005; Katsoulidou et al. 2006). IDUs are repetitively exposed to different HCV genotypes and while one could speculate that patients who have cleared infection, either spontaneously or upon treatment, carry protective immunity against reinfection, this does not seem to be the case (Dalgard et al. 2002; Backmund et al. 2004; Micallef et al. 2007; Currie et al. 2008; Bate et al. 2010; Grebely et al. 2010; Grady et al. 2012), suggesting a lack of sterilizing immunity. However the incidence of reinfection is low compared to acquisition of primary HCV infections (Mehta et al. 2002; Backmund et al. 2004; Grebely et al. 2006; Grady et al. 2012), indicating that resolved HCV infection is at least associated with partial protection. In a recent epidemiological study of IDUs living in Germany 45.2% of IDUs were HCV-RNA positive, another 27.9% were anti-HCV positive but HCV-RNA negative consistent with resolved HCV infection (Zimmermann 2012). Interestingly an additional 26.9% of IDUs were anti-HCV negative although the reported risk behavior suggests that they have been exposed to the HCV. It has been highlighted in numerous studies that both the innate and the adaptive immune response are important for spontaneous immune control of HCV (reviewed in (Rehermann 2009)) but it is largely unclear if the immune response contributes to protection against productive HCV infection in those IDUs who remain seronegative.

1.7 Humoral immune responses

The importance of antibodies in HCV infection has long been underestimated as it was demonstrated that antibody-deficient patients are able to clear HCV infection

(Semmo et al. 2006). Only recently the discussion about neutralizing antibodies in HCV infection (reviewed by (Fafi-Kremer et al. 2012)) has been revitalized.

The antibody response to HCV *in vivo* is directed against several viral proteins (Chen et al. 1999), however neutralizing Antibodies (Abs) that block HCV entry are specifically directed towards the viral envelope, particularly envelope glycoprotein E2. Functional analysis and neutralization experiments using sera from chronically HCV infected patients have demonstrated that host neutralizing responses target viral entry at a step after initial HCV binding, most likely by interrupting HCV E2-CD81 or HCV E2-SR-BI interactions, or by inhibiting membrane fusion (Haberstroh et al. 2008). Although hyper variable region I (HVRI), a region in the E2 protein displaying high genetic variability that is involved in viral entry (Bartosch et al. 2003), assembly, release of virus particles and membrane fusion (Bankwitz et al. 2010), is a primary target of neutralizing antibodies, these antibodies exhibit poor cross-neutralizing potency (Wang et al. 2011). Antibodies that demonstrate broadly neutralizing activity tend to be directed against conserved epitopes within E2 and most often inhibit the interaction between CD81 and E2 (Owsianka et al. 2001; Clayton et al. 2002; Triyatni et al. 2002; Hsu et al. 2003; Keck et al. 2008). While anti-HCV antibodies are generally detected in HCV infection, a strong and early production of neutralizing Abs during acute infection may contribute to control of the virus and facilitate viral elimination by cellular immune responses (Lavillette et al. 2005; Pestka et al. 2007; Dowd et al. 2009). In addition neutralizing Abs have been found in patients that spontaneously cleared HCV reinfection, indicating that cross-neutralizing humoral responses are able to decrease or even control viremia (Osburn et al. 2010). Despite the existence of neutralizing Abs *in vivo* the majority of patients develop chronic HCV infection, this is owed to genetically distinct, but closely related HCV variants within the quasispecies, allowing the rapid selection of mutants which are best adapted to the host environmental changes, hence the virus could persist in the presence of neutralizing Abs (von Hahn et al. 2007).

1.8   T cell responses

While the role of antibodies has long been controversial, HCV-specific T cells are critical in HCV clearance (reviewed in (Rehermann 2013). Strong CD4 helper and CD8 effector T cell responses have been observed in patients that clear HCV infection (Lechner et al. 2000). Vigorous proliferation and activation of CD4 T cells is observed in patients that later go on to resolve HCV infection (Figure 1.5A), while CD4 T cell responses are rare or absent in those who later develop chronic HCV infection (Figure 1.5B) (Missale et al. 1996). In contrast to CD4 T cells, CD8 T cells appear to be stunted in acute HCV infection (Kaplan et al. 2007), displaying decreased proliferation, IFN-γ production and cytotoxicity (Lechner et al. 2000; Thimme et al. 2001; Urbani et al. 2002) as well as increased levels of programmed death 1(PD-1) (Kasprowicz et al. 2008). However, dysfunction of CD8 T cells resolves and protective CD8 T cells expressing the IL-7 receptor α-chain, CD127, develop with the onset of CD4 activation and the decline of viral titers (Lechner et al. 2000; Thimme et al. 2001; Urbani et al. 2002).

While T cells are able to clear acute HCV infection, chronic HCV infection is associated with continuous activation yet impaired function and reduced breadth of HCV-specific T cells (Wedemeyer et al. 2002; Cox et al. 2005). Prolonged exposure to viral antigens is the main cause for the reduced frequency and impaired effector function of virus-specific CD8 T cells, summarized as T cell exhaustion (Bucks et al. 2009; Mueller et al. 2009). T cell exhaustion follows a predictable model, first T cells lose their ability to produce IL-2, followed by sequential loss of cytotoxicity and TNF-α and IFN-γ production. In addition, the intracellular expression of proapoptotic proteins such as Bsl2-interasting mediator (Bim) increases in virus-specific CD8 T cells (Larrubia et al. 2013). Accordingly T cells of chronically HCV infected individuals display increased expression of PD-1 (Penna et al. 2007; Radziewicz et al. 2007; Nakamoto et al. 2009; Bengsch et al. 2010), cytotoxic T lymphocyte antigen 4 (CTLA-4) (Radziewicz et al. 2007; Nakamoto et al. 2009), T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) (Golden-Mason et al. 2009; McMahan et al. 2010) and 2B4 (Nakamoto et al. 2009; Schlaphoff et al. 2011). Additionally the corresponding ligands are upregulated in the inflamed liver. In addition, an

inflammation-induced increase in regulatory T cell counts (Sugimoto et al. 2003) and a shift from T cell activating cytokines like interleukin-2 (IL-2) (Radziewicz et al. 2007) towards inhibiting cytokines like IL-10 and transforming growth factor-β (TGF-β) (Accapezzato et al. 2004; Alatrakchi et al. 2007) dampens virus specific T cell responses.
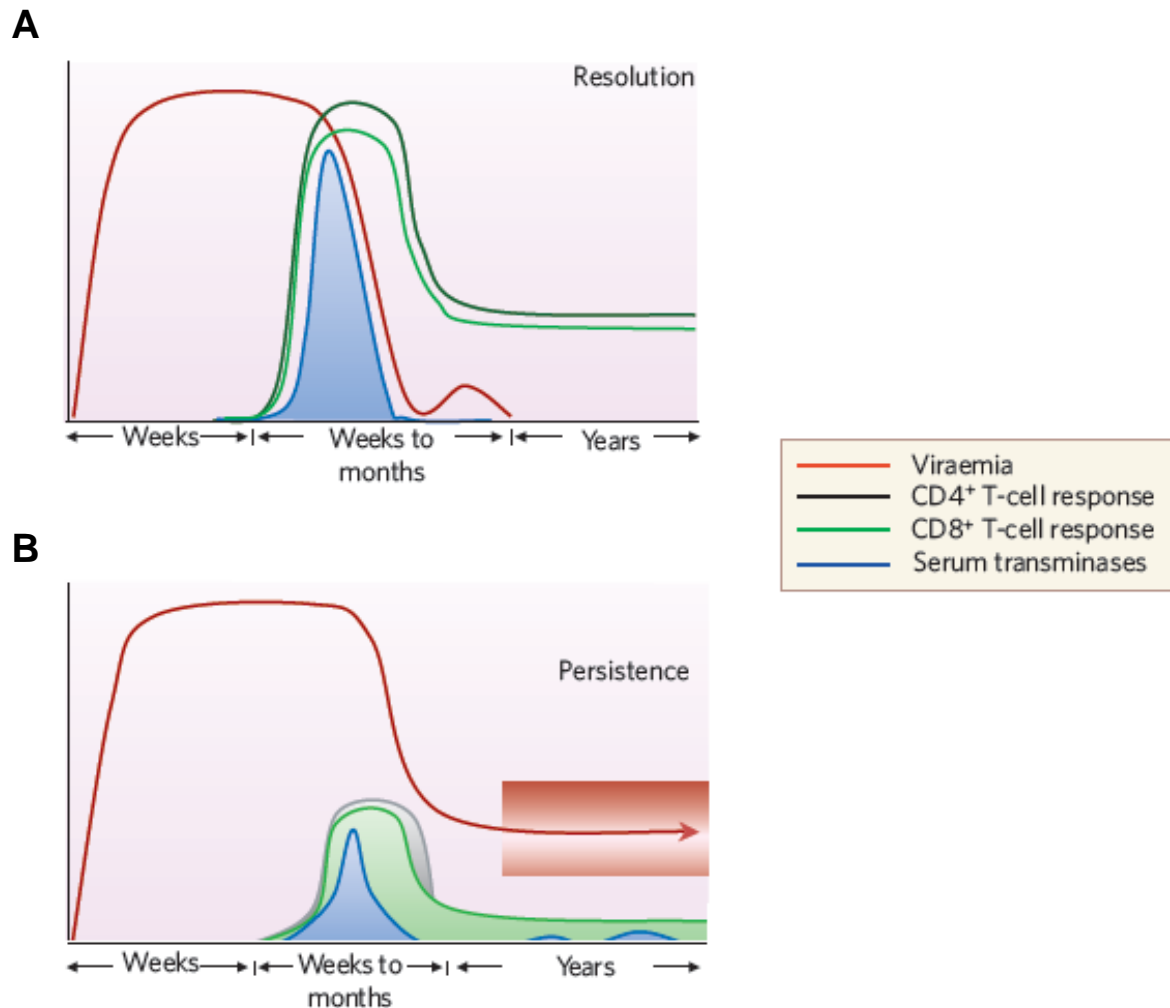
Figure 1.5: Schematic representation of the cellular immune response during acute HCV infection.
(4) Early onset and maintained CD4 and CD8 T cell responses mediate viral clearance and lead to resolution of HCV infection. (B) Week CD4 and CD8 T cell responses are unable to clear HCV infection and decrease over time allowing persistent HCV infection (Modified from (Bowen et al. 2005)).

Finally HCV recognition by T cells is further hindered by escape mutations in T cell epitopes (Aldrich et al. 1994; Chang et al. 1997; Timm et al. 2007; Neumann-Haefelin et al. 2011). While protective T cell responses tend to be directed against epitopes that do not allow escape mutations because of high costs to viral replicative fitness, viral mutations in dominant T cell epitopes are selected to establish chronic infection,

rendering existing T cell responses irrelevant. Interestingly, while novel T cell responses against HCV epitopes at later stages of infection are rare, (Cox et al. 2005) induction of T cell responses to unrelated antigens is not impaired.

1.9 HCV and innate immunity

HCV is sensed as non-self by pattern recognition receptors (PRRs) leading to activation of the innate immune system and subsequently adaptive immunity. The main receptors responsible for HCV recognition are retinoic acid inducible gene-I (RIG-I), Toll-like receptor 3 (TLR3) and protein kinase R (PKR) (reviewed by (Horner et al. 2013).

RIG-I is a cytosolic PRR sensing RNA viruses. It binds to RNA that includes an exposed 5' triphosphate and the 3' untranslated region, which is rich in poly U/UC ribonucleotides (Saito et al. 2008; Uzri et al. 2009). HCV RNA binding induces a conformational change that promotes its oligomerization and translocation from the cytosol into intracellular membranes (Saito et al. 2007; Jiang et al. 2011; Liu et al. 2012). Together with the chaperone protein 14-3-3ε and the ubiquitin ligase TRIM25, RIG-I makes up a translocon that facilitates the interaction of RIG-I with mitochondrial antiviral signaling proteins (MAVS) (Gack et al. 2007; Liu et al. 2012). The RIG-I MAVS interaction promotes the formation of a MAVS signalosome that propagates activation of downstream effector molecules, including the transcription factors interferon regulatory factor-3 (IRF-3), nuclear factor-κB (NF-κB) and a variety of proinflammatory cytokines (Loo et al. 2011) (Figure 1.6).

TLR3 is an endosomal sensor of dsRNA expressed in a number of cell types within the liver, including hepatocytes and the liver-resident macrophages called Kupffer cells (Seki et al. 2008; Wang et al. 2009). TLR3 signals are transmitted through the adaptor protein TIR-domain-containing adaptor-inducing interferon-β (TRIF), which activates IRF-3 and NF-κB for the production of type I interferons (IFNs), proinflammatory cytokines and chemokines, as well as apoptotic signaling (Salaun et al. 2006; Takeuchi et al. 2009). However, in contrast to synthetic TLR3 ligands that trigger TLR3 mediated activation within 24 h after stimulation, activation via HCV

RNA takes up to three to four days (Wang et al. 2009; Li et al. 2012). In addition, cytokine induction profiles after TLR3 activation using the TLR3 agonist polyinosine-cytosine (poly I:C) and HCV RNA differ, suggesting HCV-TLR3 interaction has some level of specificity (Wang et al. 2009; Li et al. 2012). During HCV infection TLR3-mediated signaling could serve as a secondary innate immune detection or surveillance system for uninfected cells after RIG-I detection of HCV, and it could be involved in setting up an antiviral state within regions of the liver or in chemokine induced T cell recruitment (Wang et al. 2009) (Figure 1.6).

PKR is a dsRNA–binding protein whose kinase activity can be induced by binding to HCV dsRNA to phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF2α). However, this only downregulates host mRNA translation, as HCV is independent of eIF2α due to the lack of a 5' cap structure (Koev et al. 2002; Garaigorta et al. 2009; Shimoike et al. 2009; Arnaud et al. 2010). It is known that PKR binding of HCV dsRNA also activates a kinase-independent signal transduction cascade that drives induction of specific IFN-stimulated genes (ISGs) and IFN-β production by signaling through MAVS, tumor necrosis factor (TNF) receptor-associated factor 3, IRF and NF-κB, all before RIG-I activation (Kumar et al. 1997; McAllister et al. 2009; Arnaud et al. 2010) (Figure 1.6).

1.9.1 HCV evasion of innate antiviral immunity

The NS3-NS4A protease is a central component of the HCV innate immune evasion strategy. It is anchored to intracellular membranes through the NS4A transmembrane domain and an amphipathic α-helix at the NS3 N-terminus that facilitates membrane association and cleavage of membrane anchored substrates (Brass et al. 2008; Horner et al. 2012). Hence NS3-NS4A targets and cleaves MAVS from intracellular membranes and prevents signal transduction (Foy et al. 2003; Foy et al. 2005; Meylan et al. 2005; Baril et al. 2009; Loo et al. 2011) (Figure 1.6 (1,3)). This cleavage event prevents activation of the RIG-I pathway and downstream signaling events including IFN induction. In addition, the NS3-NS4A protease also proteolytically targets TRIF (Li et al. 2005)(Figure 1.6 (4)), the TLR3 signaling adaptor proteins, thus

preventing TLR3 signaling as well. In combination theses cleavage events abrogate IFN induction and support progression to chronic infection.
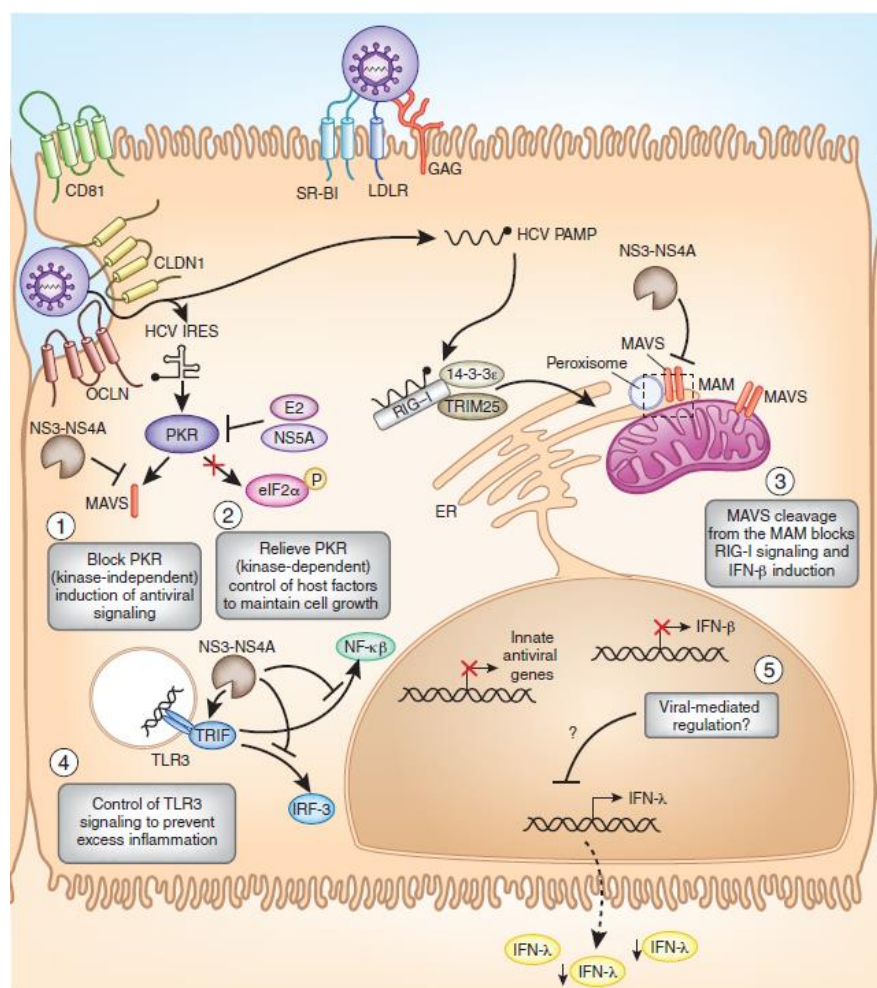
Figure 1.6: HCV recognition by and evasion from innate immunity.
HCV is sensed by the PRRs RIG-I (3), TLR3 (4) and PKR (1,2) and evades innate immunity by cleavage of host factors. The proposed regulation is depicted above. The HCV NS3-NS4A protease cleaves the signaling adaptors MAVS (indicated by the dashed box) and TRIF to inactivate PKR (1), RIG-I (3) and TLR3 (4) signaling pathways to prevent induction of immunomodulatory innate antiviral genes and IFN-β, allowing for HCV replication. HCV infection control of IFN-λ induction is not yet defined (5); HCV E2 and NS5A proteins inactivate PKR-dependent activation of the host translation factor eIF2α to reactivate protein translation during infection (2)(Horner et al. 2013).

1.10 Influence of host genetics on HCV infection outcome

Several factors influence spontaneous clearance of HCV infection as well as treatment outcome including HLA-type, ethnicity, gender, age and obesity. Furthermore, high baseline levels of ISGs predict an unfavorable outcome of HCV therapy (Kau et al. 2008). Single nucleotide polymorphisms (SNPs) upstream of the

IFNL3 (also referred to as *IL28B*) locus that can predict both successful clinical outcome to HCV therapy (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009; Rauch et al. 2010) and spontaneous resolution have been identified (Thomas et al. 2009; Rauch et al. 2010). Theses SNPs associate with altered mRNA expression of *IFNL3*, which encodes the anti viral cytokine IFNL3, which suggests that IFNL3 expression levels are probably associated with HCV clearance and response to therapy (Suppiah et al. 2009; Tanaka et al. 2009; Dill et al. 2011). In fact, is has been shown that the unfavorable SNP results in less IFNL3 expression in the liver, peripheral blood mononuclear cells and whole blood (Suppiah et al. 2009; Tanaka et al. 2009; Fukuhara et al. 2010; Dill et al. 2011; Langhans et al. 2011). A recent study showed that patients with the unfavorable *IFNL3* genotype have depressed innate immune function, particularly with respect to NK cells (Naggie et al. 2012), suggesting the decreased expression of IFNL3 affects immunity and therefore clearance of HCV. Similar to the *IFNL3* genotype, the *KIR2DL3* and *HLA-C1* genotype have been associated with spontaneous resolution of HCV infection (Khakoo et al. 2004) as well as resistance to HCV infection (Knapp et al. 2010). Although it is well known that the *KIR2DL3* gene encodes for the inhibiting natural killer (NK) cell receptor KIR2DL3 and that HLA-C1 molecules are the respective ligands for this receptor, the mechanisms underlying this protective effect remain elusive. However, different NK cell phenotypes have repeatedly been associated with HCV infection outcome.

1.11 Natural killer cells

1.11.1 Natural killer cells in viral infections

NK cells constitute a population of bone marrow derived, low-density, large granular lymphocytes that make up 10–15% of the Peripheral Blood Mononuclear Cell (PBMC) and are strongly enriched in the liver (Doherty et al. 2000). They lack the normal B and T cell lineage markers (CD19 and CD3) and are usually characterized by the expression of CD56, the neural cell adhesion molecule, and CD16, the Fcγ receptor III, which mediates antibody dependent cellular cytotoxicity (ADCC).

They are able to spontaneously kill virally infected and tumor cells without prior sensitization by surveillance of danger signals including down regulation of major

histocompatibility complex (MHC) class I molecules ("missing self") (Ljunggren et al. 1990) and upregulation of MHC class I homologues ("induced self") (Raulet 2003) as well as direct recognition of pathogen associated molecules (Yokoyama et al. 2004; Lanier 2005). NK cells are able to mediate viral control in various ways. They can directly kill infected cells by release of granzyme and perforin from cytotoxic granules (Farag et al. 2006) or limit viral replication by production of cytokines like IFN-γ (Moretta et al. 2002). In addition, they are able to recruit other lymphocytes to the site of infection, including T cells (Zingoni et al. 2005; Welsh et al. 2013) and dendritic cells (DC) (Fernandez et al. 2002).

1.11.2 NK cell receptors

NK cell activation is controlled by a complex set of activating and inhibiting receptors on the NK cell surface. The receptor repertoire varies between patients and is known to influence HCV infection outcome. In humans the majority of NK cell receptors can be grouped into three receptor families.

1.11.2.1 The killer cell immunoglobulin-like receptor (KIR) family

KIRs are type I integral membrane glycoproteins that are usually expressed as monomers on the cell surface (Lanier 1998; Long 1999). KIR receptors are subclassified based on the number of Ig domains of the extracellular part (KIR2D/3D) and the length of the cytoplasmic tail. The ones with long cytoplasmic tails (KIR2D/3DL) have two immunoreceptor tyrosine-based inhibition motifs (ITIMs) and are inhibiting receptors. Those with a short cytoplasmic tails (KIR2D/3DS) associate with a dimer of the immunoreceptor tyrosine-based activation motif (ITAM) bearing adapter protein DAP12 and mediate activating signals (Lanier et al. 1998) (Figure1.7A). The receptors are further numbered differently if they show more than 2% sequence divergence within the group. The ligands for KIR receptors are HLA molecules.

1.11.2.2 NKG2/CD94 receptors

NKG2/CD94 receptors are type II C-type, lectin-like, integral membrane glycoproteins that are expressed on the cell surface as a heterodimer with CD94 (Figure 1.7B). CD94 has no signaling properties and only serves to stabilize NKG2 on the cell

surface. Four distinct genes A/B, C, D, E/H, and F encode the NKG2 receptors (Lanier 1998; Long 1999; Diefenbach et al. 2001). Only the NKG2A/CD94 receptor has a long cytoplasmic tail containing two ITIMs, thus mediating inhibiting signals to the NK cell. The other members of the NKG2 family mediate activating signals and associate with DAP12 (Lanier et al. 1998). NKG2 receptors bind HLA-E (Borrego et al. 1998; Braud et al. 1998; Lee et al. 1998), whose expression requires peptides derived from signal sequences of HLA-A, -B, -C, and –G (Leibson 1998).

NKG2D represents an exception within the NKG2 family. Unlike the other family members it forms a homodimer on the cell surface (Figure 1.7B). Its ligands are the stress induced non-classical MHC class I molecules MIC-A/B and ULBP1-4. Upon binding NKG2D mediates activating signals to the NK cell (Bauer et al. 1999; Wu et al. 1999).
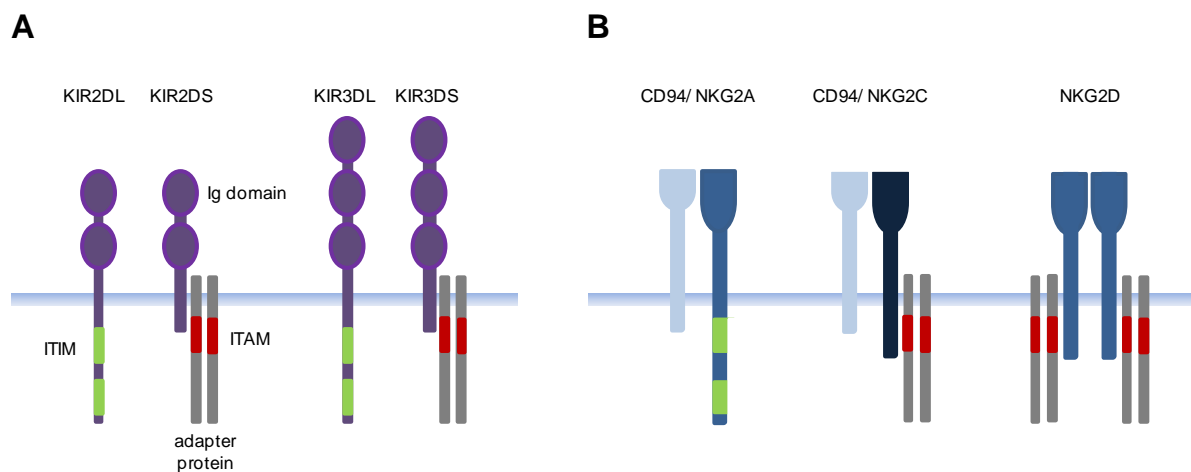
Figure 1.7: The KIR and NKG2/CD94 receptor families.
(4) KIR receptors are named KIR2D (left) or KIR3D (right) according to the number of IG domains expressed. Inhibiting KIR receptors have a long cytoplasmic tail with two ITIM motive (green). Activating KIRs have a short cytoplasmic tail and associate with ITAM (red) containing adapter proteins. (B) Members of the NKG2 receptor family usually associate with a CD94 molecule. NKG2D represents an exception forming hetero dimmers. Activating members of the NKG2 family associate with ITAM bearing adapter proteins. The inhibiting NKG2A receptor has a cytoplasmic tail containing ITIMs.

1.11.2.3   Natural cytotoxicity receptors (NCR)

NKp30, NKp44, and NKp46 are NCR (Figure 1.8). They belong to the Ig superfamily and trigger NK cell activation upon engagement. NKp46 and NKp30 are expressed on resting and activated NK cells, whereas NKp44 is expressed on activated NK cells

(Biassoni et al. 2001; Moretta et al. 2001). NKp46 binds the sialic acid-binding glycoproteins, e.g., hemagglutinin and hemagglutinin-neuraminidase of the influenza and parainfluenza viruses, respectively (Mandelboim et al. 2001). NKp44 bind to the E2 protein of West Nile and Dengue virus (Hershkovitz et al. 2009). Other ligands for NCRs are not conclusively defined.
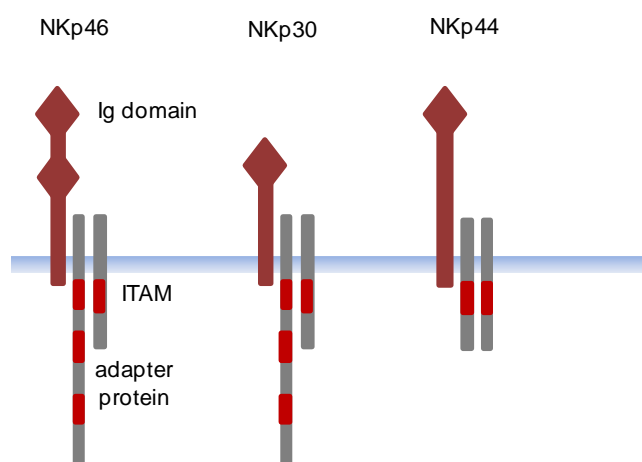
Figure 1.8: Schematic drawing of NKp46, NKp30 and NKp44.
NKp46, NKp30 and NKp44 are activating NK cell receptors associated with ITAM bearing adapter proteins.

1.11.3 NK cell differentiation

Based on the expression of CD56 and CD16 NK cells are subdivided into three distinct subsets. About 10% of NK cells in the peripheral blood are CD56^{bright} CD16^{-}. This immunoregulatory subset produces a wide range of cytokines and chemokines after stimulation, but its ability to spontaneously kill target cells is poor. In contrast, the remaining 90% of NK cells, the CD56^{dim}CD16^{+} cytotoxic NK cell subset, produce lower cytokine levels, but possess an abundance of cytolytic granules and can spontaneously lyse susceptible target cells (Poli et al. 2009). The third subset, CD56^{-}CD16^{+} NK cells, are rare but were described to be expanded in HIV infection (Hu et al. 1995). These NK cells display impaired functionality and are believed to be anergic NK cells (Hu et al. 1995; Alter et al. 2005).

NK cells differentiate from CD56^{bright} to CD56^{dim} to CD56^{-} NK cells (Ouyang et al. 2007; Romagnani et al. 2007). While almost all CD56^{bright} NK cells express NKG2A on their cell surface, differentiation of NK cells from CD56^{bright} to CD56^{dim} is accompanied by the sequential loss of NKG2A and acquisition of KIRs, CD57 and

NKG2C (Beziat et al. 2010; Bjorkstrom et al. 2010; Beziat et al. 2012). It is well established that KIRs, in conjunction with NKG2A, play a major role in NK cell education that determines whether NK cells will end up competent or hyporesponsive. The mechanisms underlying this education are still debated. However, the most likely model to date is a hypothesis proposing that the engagement of self-MHC-specific inhibitory receptors (KIRs) directly renders NK cells functional, "licensing" them so to speak.

1.12 NK cells in HCV infection

1.12.1 NK cells in acute HCV infection

The phenotype of NK cells has repeatedly been associated with HCV infection outcomes. Increased IFN-γ production, cytotoxicity and degranulation have been described in acute HCV infection (Amadei et al. 2010; Pelletier et al. 2010), however NK cell activation did not correlate with subsequent infection outcome. Phenotypical changes have also been observed in acute HCV infection. Amadei et al. report increased frequencies of NKG2D expressing NK cells, yet this phenotype is not associated with a specific infection outcome (Amadei et al. 2010). Alter et al. on the other hand report lower frequencies of NKp46$^+$ and NKp30$^+$ NK cells in acutely infected patients that go on to clear infection (Alter et al. 2011). These findings are somewhat counterintuitive as recent reports associate high levels of NKp30 (Golden-Mason et al. 2010) and NKp46 (Sivori et al. 1999; Kramer et al. 2012) with HCV clearance. Alter et al. suggest that this downregulation might be explained by earlier vigorous NK cell activation and subsequent downregulation of activating NK cell receptors (Alter et al. 2011).

1.12.2 NK cells in chronic HCV infection

While data about NK cells in acute HCV infection is scarce, numerous studies on NK cells in chronic HCV infection exist. Several studies report reduced NK cell frequencies in the peripheral blood of chronically HCV infected patients compared to healthy controls (Meier et al. 2005; Morishima et al. 2006; Bonorino et al. 2009; Oliviero et al. 2009) as well as decreased intrahepatic NK cell levels (Kawarabayashi et al. 2000; Deignan et al. 2002; Bonorino et al. 2009). Altered subset distribution

characterized by decreased frequencies of CD56^{dim} and/or increased frequencies CD56^{bright} NK cells is a consistent finding (Golden-Mason et al. 2008; Bonorino et al. 2009). Several NK cell receptors have been reported to be differentially expressed in HCV infection (reviewed in (Cheent et al. 2011)). However, conflicting data on the NK cell phenotype in chronic HCV infection exists. This variance may arise from differences in methodology, control groups used, the use of fresh or frozen blood samples and small sample sizes. However, one of the most consistent findings links an expansion of NKG2A^{+} NK cells to chronic infection (Jinushi et al. 2004; Nattermann et al. 2005; Nattermann et al. 2006; Ahlenstiel et al. 2010), which suggests inhibition of NK cell function. Several lines of evidence suggest skewing or polarization of NK cell function away from IFN-γ production towards cytotoxicity (Oliviero et al. 2009; Ahlenstiel et al. 2010; Dessouki et al. 2010; Mondelli et al. 2012). Insufficient IFN-γ responses may result in increased viral replication, as IFN-γ has direct antiviral properties and can control viral replication (Boehm et al. 1997; Rotondi et al. 2011) while enhanced cytotoxicity may promote liver damage (Ahlenstiel et al. 2010).

Lately, new data from the lymphocytic choriomeningitis virus (LCMV) mouse model suggested that NK cells kill activated T cells in chronic viral infection. Lang et al. demonstrated that NK cells have a negative impact on the development of T cell immunity during LCMV infection. NK cell deficient mice exhibited a higher virus-specific T cell response. In addition, NK cell depletion in wild type mice caused increased T cell immunity and viral clearance (Lang et al. 2012). A similar study in the LCMV model by Waggoner et al. describes a three-way interaction, whereby activated NK cells cytolytically eliminate activated CD4 T cells, which affects CD8 T cell function and exhaustion. At high virus doses, NK cells prevented fatal pathology while enabling T cell exhaustion and viral persistence, but at medium doses NK cells facilitated lethal T cell-mediated pathology, thus suggesting that NK cells act as regulators of T cell immunity (Waggoner et al. 2012). Importantly, similar findings were made in human HBV infection. Peppa et al. found that NK cells can rapidly eliminate HBV-specific T cells in a contact-dependent manner. CD8 T cells in the liver microcirculation were visualized making intimate contact with NK cells. These

findings demonstrate that NK cells can negatively regulate antiviral immunity in chronic HBV infection illustrating a potential novel mechanism of T cell tolerance in the human liver (Peppa et al. 2013). Taken together this data suggests that NK cell activation might be detrimental in chronic viral infection and supports ongoing chronicity, while inactive NK cells allow vigorous T cell responses and viral clearance.

1.12.3 NK cells in HCV exposed seronegative individuals

Khakoo et al. have described a strong genetic association between homozygosity for the NK cell receptor gene *KIR2DL3* and its ligand *HLA-C1* group alleles and spontaneous clearance of HCV infection in IDUs (Khakoo et al. 2004). Interestingly, the same genetic association was confirmed in HCV seronegative IDUs (Knapp et al. 2010; Knapp et al. 2011), while a phenotypic analysis of NK cells described increased levels of NKp30 in HCV seronegative IDUs (Golden-Mason et al. 2010). Importantly, a recent study demonstrated activation of the NK cell compartment after needle stick exposure to HCV without subsequent seroconversion (Werner et al. 2013), confirming that NK cells act against HCV prior to induction of adaptive immune responses. Taken together with reports that IL-6 and IL-8, modulators of innate immunity, are upregulated in serum of HCV exposed seronegative individuals (Warshow et al. 2012), this data indicates an important role of NK cells in early immune control of HCV infection.

1.13 Aim of the study

In a recent epidemiological study of IDUs living in Germany 45.2% of IDUs were HCV-RNA positive, another 27.9% were anti-HCV positive but HCV-RNA negative consistent with resolved HCV infection and the remaining 26.9% of IDUs were anti-HCV negative (Zimmermann 2012). The frequently reported shared use of drug injection equipment suggests that most IDUs in cohorts with high HCV prevalence rates are exposed to HCV (Scherbaum et al. 2009; Pouget et al. 2012). Indeed, the existence of HCV-specific CD8 T cell responses in a subset of seronegative IDUs supports prior exposure to HCV (Thurairajah et al. 2008; Cameron et al. 2013). Studies by Khakoo et al. and Knapp et al. suggest that KIR2DL3 is the main player in refering resistence to HCV infection (Khakoo et al. 2004; Knapp et al. 2010). These studies show a strong corellation between homozygosity for *KIR2DL3* and *HLA-C1*

and spontaneous clearance of HCV as well as resistence to HCV infection. As KIR2DL3 is not exclusively expressed on NK cells (Paust et al. 2010) and those studies refered to the genotype alone and phenotypical analysis are not included, the aim of this sudy is to analyze NK cells of seronegative IDUs in more detail to answer the following questions:

- Is there an NK cell phenotype that can mediate "a state of natural resistence" to HCV infection?

- What is the role of KIR2DL3 in mediating resistance to HCV?

2 Materials

2.1 Chemicals and reagents

BD^{TM} Comp beads, anti mouse Ig κ	Becton Dickinson
Biocoll Separating Solution	Biochrome
Bright-Glo™ Luciferase Assay System	Promega
Dimethylsulfoxid (DMSO)	Roth
Dulbecco's Phosphate-Buffered Saline, 1X (DPBS)	Gibco
Ethanol	Sigma-Aldrich
FACS-Clean	Becton Dickinson
FACS-Flow	Becton Dickinson
FACS-Rinse	Becton Dickinson
Glo Lysis buffer	Promega
IC Fixation Buffer	eBioscience
Isopropanol	Sigma-Aldrich
Permeabilization Buffer (10X)	eBioscience

2.2 Cell culture media and additives

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1M	PAA
Brefeldin A (BFA)	Sigma Aldrich
Dulbecco's Modified Eagle's Medium (DMEM)	PAA
Fetal bovine serum (FBS)	Biochrome
G-418	Biochrome
Interferon (IFN)- α	Promokine
Non-essential amino acids	PAA
p815-specific antibody	Abcam
Penicillin/Streptomycin (100X)	PAA
RPMI 1640 + L-Glutamin	PAA
Trypsin/ EDTA (100X)	PAA
α-Galactosylceramide (α-GalCer)	Funakoshi

2.3 Eukaryotic cell lines

2.3.1 Human acute myelocytic leukemia cell line K562 (K562)

K562 cells are human immortalized myelogenous leukemia cells obtained from a 53 year old female patient with chronic myelogenous leukemia undergoing blast crisis (Lozzio et al. 1975). Due to a lack of MHC molecules on their surface they are easily killed by NK cells (Lozzio et al. 1979).

2.3.2 K562 HLA-E

K562 HLA-E cells are K562 cells stably transfected with *HLA-E*01033* (Nattermann et al. 2005).

2.3.3 LCL721.221 cells (221)

LCL721.221 cells are a subline of the human B lymphoblastoid cell line (LCL) 721 with γ-ray induced HLA antigen loss (Shimizu et al. 1989).

2.3.4 Primary human hepatocytes

Primary human hepatocytes were isolated from liver tissue of patients undergoing liver transplantation and obtained from the department of gastroenterology.

2.3.5 Murine lymphoblast-like mastocytoma cell line p815 (p815)

Murine lymphoblast-like mastocytoma cell line p815 cells are used to assess antibody-dependent cell-mediated cytotoxicity of NK cells. For that purpose p815 cells were coated with p815 specific antibody and subsequently used as target cells for NK cells.

2.4 Cell culture media composition

K562, LCL 721.221, p815, PBMC (R10)	RPMI 1640
	10% FBS
	100 U/mL penicillin
	100 µg/mL streptomycin
	10 mM HEPES

K562 HLA-E   RPMI 1640

                                             10% FBS

                                             100 U/mL penicillin

                                             100 µg/mL streptomycin

                                             10 mM HEPES

                                             300 U/mL G-418

Freezing medium   FBS

                                             10% DMSO

Primary human hepatocytes (PHH)   DMEM/ Ham´s F12

                                             10% FBS

                                             100 U/mL penicillin

                                             100 µg/mL streptomycin

2.5   Commercial Kits

3' IVT Express Kit	Affymetrix
ArC^{TM} Bead Kit	Invitrogen
EasySep™ Human NK Cell Enrichment Kit	Stemcell Technologies
EasySep™ Human PE Positive Selection Kit	Stemcell Technologies
Experion RNA StdSens Analysis Kit	Bio-Rad
Liaison^{TM} CMV IgG II	DiaSorin
LightCycler FastStart DNA Master^{PLUS} HybProbe PCR Kit	Roche
miRNeasy Micro Kit	Quiagen
Qiamp DNA Blood Mini Kit	Quiagen
QuantiTect SYBR Green RT-PCR Kit	Quiagen
Rneasy Mini Kit	Quiagen

2.6   Antibodies for flow cytometry

All Antibodies used in this study were anti-human antibodies and stored at 4 °C. The concentrations applied for the antibodies utilized in this study are summarized in table 2.1.

Table 2.1: Fluorochrome conjugated antibodies for flow cytometry

Specificity	Fluorochrom	Concentration; μl/ 10^6 cells	Company
Blue Viability Dye	UV exitation	1	Invitrogen
CD1d	Allophycocyanin (APC)	0.125 μg/μl 1	eBiosiences
CD3	Pacific Blue	0.2 μg/μl 2	Becton Dickinson
CD3	Phycoerythrin (PE)	2	Becton Dickinson
CD14	Pacific Blue	0.2 μg/μl 2	Becton Dickinson
CD16	APC-Cyanine7 (APC Cy7)	2	Becton Dickinson
CD19	Pacific Blue	2	Becton Dickinson
CD56	PE-Cy7	2	Becton Dickinson
CD57	(Fluorescein isothiocyanate) FITC	0.2 μg/μl 1	eBiosiences
CD107a	Alexa 700	1 μg/μl 2	Becton Dickinson
CD127	FITC	0.025 μg/μl 1	eBiosiences
HLA-E	PE	0.1 μg/μl 1	eBiosiences
IFN-γ	FITC	0.1 μg/μl 1	eBiosiences
IFN-γ	PerCP Cy5.5	0.012 μg/μl 5	eBiosiences
KIR2DL3	FITC	0.025 μg/μl 5	R&D Systhems
KIR2DL3	PE	10	R&D Systhems
Mouse IgG1 Iso Control	PE	1	R&D Systhems
Mouse IgG2A Iso Control	FITC	5	R&D Systhems
NKG2A	APC	5	Beckman coulter
NKG2A	PE	5	Beckman coulter

NKG2C	PE	0.025 µg/µl 5	R&D Systhems
Vα24	FITC	5	Beckman coulter
Vβ11	APC	5	Beckman coulter
CD1d Tetramer	APC	0.2	ProImmune
CD1d Tetramer Iso control	APC	0.2	ProImmune

2.7 Consumables and Equipment

Cell culture flasks, T25, T75	Greiner Bio-One
Cell culture plates (6-, 12-, 24-, 48-, 96-well)	Greiner Bio-One
Centrifuge 5415C	Eppendorf
Centrifuge 5415R	Eppendorf
Cryo tubes, 2.0 ml	Greiner Bio-One
EasySep purple magnet	Stemcell technologies
Glomax multi detection system	Promega
KX-21N	Sysmex
Leucosep™ tubes, 50 mL	Greiner Bio-One
Liaison XL	DiaSorin
LightCycler 2.0	Roche
Megafuge 1.0R	Heraeus
Megafuge 40R	Thermo Scientific
Microscope Primo Vert	Zeiss
Microscope TMS	Nikon
Mr. Frosty freezing container	Thermo Scientific
Polystyrene round-bottom tubes, 5 mL	BD Falcon™

Flow cytometer

FACS Calibur   Becton Dickinson

FACS Canto II   Becton Dickinson

LSR II   Becton Dickinson

2.8   Software and Webpages

FlowJo 7.6   Tree Star, Inc. 1997-2008

GraphPad Prism   GraphPad Software, Inc

Microsoft Office   Microsoft

3   Methods

3.1   Cell culture

3.1.1   Thawing of cells

One vial of frozen cells (K562, K562 HLA-E, P815, LCL721.221) was taken out of the liquid nitrogen and quickly thawed in a 37 °C water bath. The cells were then resuspended in 10 mL PBS and centrifuged at 528 xg for 7 min. After the washing step the cells were taken up in 20 mL of the appropriate media and placed into a T75 cell culture flask.

3.1.2   Passaging of suspension cells

Suspension cells (K562, K562 HLA-E, P815, LCL721.221) were thoroughly resuspended and placed into the new flask in the designated concentration.

3.1.3   Freezing of cells

Cells were washed in 10 mL PBS and centrifuged at 528 xg for 7 min. The supernatants were removed and the cells were transferred to a cryo tube in 1 mL freezing medium (FCS containing 10% DMSO). The cryo tubes were transferred to a Mr. Frosty freezing container filled with isopropanol and kept at -80 °C over night allowing a temperature drop of approximately 1 °C/min. The cells were transferred to liquid nitrogen the following day.

3.2 Immunological Methods

3.2.1 Isolation of PBMCs (peripheral blood mononuclear cell) from peripheral blood

To isolate PBMCs from peripheral blood Ficoll density centrifugation was used. Differential migration during centrifugation results in the separation of cell types into different layers. The bottom layer contains Ficoll-aggregated red blood cells. Immediately above is a diffuse layer containing mostly granulocytes and unbound Ficoll. Due to a slightly lower density, the lymphocytes sediment at the interface between the Ficoll and uppermost plasma layer.

For PBMC isolation 15 mL of Biocoll Separating solution was transferred to a 50 mL Leucosep tubes and centrifuged for 1 min at 367 xg to allow the separating solution to pass through the filter. Afterwards 30 mL of blood were carefully placed in the tube and centrifuged for 10 min at 790 xg. After centrifugation the upper layer, containing lymphocytes and granulocytes, was transferred to a fresh 50 mL Falcon tube and washed in a total volume of 40 mL PBS. The cells were centrifuged at 652 xg for 8 min. After two additional washing steps the cells were transferred to freezing medium and placed in a Mr. Frosty freezing container at -80°C. The cells were transferred to liquid nitrogen the following day.

3.2.2 Flow cytometric identification and characterization of NK cells

Frozen PBMCs were removed from the liquid nitrogen tank and quickly thawed in a 37 °C water bath and taken up in 10 mL PBS. After three washing and centrifugation steps (528 xg; 7 min) PBMCs were counted using the KX-21N cell counter. $1*10^6$ PBMSs were transferred to Polystyrene round-bottom tubes and centrifuged at 367 xg for 5 min. Subsequently the cells were stained for 15 min at room temperature using 1 µl Blue viability dye and 1 mL PBS per tube. After an additional washing step the cells were stained with fluorochrome-conjugated antibodies against cell surface proteins. To identify NK cells, PBMCs were stained with Pacific Blue labeled antibodies against CD3, CD14 and CD19, allowing the exclusion of T cells, monocytes and B cells. PMBCs were further stained with antibodies against CD16 (APC-Cy7) and CD56 (PE-Cy7) allowing further characterization of NK cells.

Additional fluorochrome-conjugated antibodies against surface proteins were added according to requirements. A master mix containing all antibodies of interest was prepared and equally distributed to each sample. The antibody cell mix was allowed to incubate at room temperature for 15 min. After incubation cells were washed using PBS and fixated at room temperature for 15 min using 100 µl IC Fixation Buffer per tube. Fixated cells were washed with PBS and analyzed using a LSRII flow cytometer.

3.2.3 Flow cytometric functional characterization of NK cells

To test NK cell functionality, frozen PBMCs were removed from the liquid nitrogen tank and quickly thawed in a 37 °C water bath and taken up in 10 mL PBS. After three washing and centrifugation steps (528 xg; 7 min) PBMC were counted using the KX-21N cell counter. $1*10^6$ PBMCs were transferred to Polystyrene round-bottom tubes.

In parallel NK cell target cells (K562, K562 HLA-E, 221) were washed three times with PBS and brought to a concentration of $1*10^6$ cells/mL PBS. P815 cells were washed and incubated with p815-specific antibody at 1mg/mL for 30 min. After an additional washing step to remove unbound antibody, cells were brought to a concentration of $1*10^6$ cells/mL PBS. 100 µl of target cell solution was transferred to the PBMC containing Polystyrene round-bottom tubes to establish an effector target ratio of 1:10. After centrifugation the supernatants were removed and the cells were resuspended in 1 mL R10, containing 10 ng/mL Brefeldin A and 2 µl AlexaFlour700 labeled CD107a specific antibody.

After incubation for 5 h at 37 °C the cells were stained using Blue Viability dye for 15 min at room temperature and washed with PBS. The cell suspension was than incubated with antibodies against surface proteins (15 min, room temperature) washed with PBS and subsequently fixated. After fixation the cells were washed twice with 1x Permeabilization Buffer. After permeabilization PBMCs were stained for intracellular IFN-γ, previously retained by Brefeldin A, using IFN-γ specific antibody label with FITC or PerCP Cy5.5. The cells were incubated for 15 min at room

temperature. After incubation cells were washed with PBS and analyzed using a LSRII flow cytometer.

3.2.4 Enrichment of NK cells

PBMCs were taken out of the nitrogen tank and thawed as described above. Cells were counted using the KX-21N cell counter and $1*10^8$ PBMCs at a concentration of $5*10^7$ cells/mL (2 mL) in PBS containing 2% FCS were used to isolate NK cells with the Stemcell Technologies EasySep™ Human NK Cell Enrichment Kit. In accordance with the provided protocol 100 µl of EasySep™ Human NK Cell Enrichment Cocktail was added to the cell suspension and incubated at room temperature for 10 min. Subsequently 200 µl of vortexed EasySep™ Magnetic particles were added and incubated at room temperature for 10 min. After that the Polystyrene round-bottom tube containing the suspension was transferred to the EasySep™ Magnet and incubated for 2.5 min. After incubation the contents of the tube was poured into a fresh 15 mL falcon tube, with the tube remaining in the magnet during the procedure. This ensured that only NK cells were transferred to the new tube, while the non-NK cells were held back magnetically. The empty tube was refilled with 2.5 mL PBS containing 2% FCS and the procedure was repeated twice. The number of NK cell enriched cells was assessed using the KX-21N cell counter. The tube containing the non-NK cells was discarded.

3.2.5 Enrichment of NKG2A positive NK cells

NKG2A positive NK cells were enriched using the Stemcell Technologies EasySep™ Human PE Positive Selection Kit. In accordance with the provided protocol the previously prepared NK cell enriched cells were centrifuged at 528 xg for 5 min. After discarding the supernatant the cells were resuspended in 100 µl PBS containing 2% FCS and incubated with 10 µl species-specific FcR blocking antibody and PE labeled NKG2A specific antibody (5 µl/ $1*10^6$ cells). After 15 min of incubation, 20 µl of Easy Sep PE Selection Cocktail were added and incubated for 15 min. Subsequently 10 µl of resuspended EasySep Magnetic Nanoparticles were added and incubated for 10 min. In the next step the Polystyrene round-bottom tube was transferred to the EasySep™ Magnet and incubated for 5 min. After incubation the

contents of the tube was poured into a fresh 15 mL falcon tube, with the tube remaining in the magnet during the procedure, ensuring that the magnetically labeled NKG2A positive cells remained in the tube while the NKG2A positive cell depleted fraction was transferred to the new tube. Afterwards the tube was refilled with 2.5 mL PBS containing 2% FSC and the procedure was repeated twice. After the third separation step the magnetically labeled NKG2A positive cells were resuspended in 7.5 mL PBS and the cell numbers of the NKG2A positive and negative fraction was assessed using the KX-21N cell counter. All incubation steps were carried out at room temperature.

3.2.6 Treatment of primary human hepatocytes (PHHs) with IFN-α

PHHs were obtained from the Department of Gastroenterology and Hepatology at the University Hospital Essen. When received, cells were seeded in 24-well plates, each well containing 500.000 cells in 500 µl medium. The cells were treated with 100 IU/mL IFN-α for a period of 24 h. Untreated controls were incubated with medium alone. After 24 h medium was removed and the adherent PHHs were washed twice with sterile PBS. Afterwards each well was treated with 200 µl trypsin for 2 min at 37 °C. The no longer adherent cells were taken up in 500 µl PBS per well and transferred to Polystyrene round-bottom tubes and centrifuged at 367 xg for 5 min. The supernatants were removed. For staining, untreated cells or cells treated with IFN-α from four wells were pooled and incubated with 1 µl HLA-E specific antibody labeled with PE for 15 min at room temperature. After incubation cells were washed using PBS and fixated at room temperature for 15 min using 100 µl IC Fixation Buffer. Fixated cells were washed with PBS and analyzed using a FACS Calibur flow cytometer.

3.2.7 Induction of HLA-E expression on K562 HLA-E cells

K562 HLA-E cells were incubated with 100 µmol/L $core_{35-44}$ peptide (amino acid sequence: YLLPRRGPRL) at 28 °C over night. HLA-E expression was assessed using flow cytometry. The cells were stained using a PE labeled HLA-E specific antibody for 15 min at room temperature, washed and fixed using 100 µl IC Fixation Buffer. Fixed cells were washed and resuspended in PBS. Analysis was performed using a FACS Calibur flow cytometer.

3.3 Microarray analysis of HLA-E expression in liver tissue from HCV- and HBV-infected patientsa

Microarray analysis was carried out by the Department of Gastroenterology and Hepatology at the University Hospital Essen.

Liver biopsies from 51 patients with chronic HCV and from 22 patients with HBV infection were collected in the Department of Gastroenterology and Hepatology at the University Hospital Essen. All diagnostically indicated needle biopsies were taken within the PROFILE biomarker project (Kohl et al. 2013) Briefly, 5 to 8 mg liver tissue were homogenized with the TissueRuptor in QIAzol Lysis Reagent to isolate total RNA using the miRNeasy Micro Kit according to the protocols of the manufacturer. RNA integrity was determined on the Experion Automated Electrophoresis System using the Experion RNA StdSens Analysis Kit. RNA samples were preprocessed with the 3' IVT Express Kit and then hybridized on Human Genome U219 16-Array Plates using the GeneTitan MC Instrument according to the manufacturer's instructions. Robust multi-array average (RMA) normalization and data analysis was carried out with the Expression Console Software (version 1.2.1, Affymetrix).

3.4 Isolation and stimulation of primary human hepatocytes and analysis of HLA-E expression

Isolation and stimulation of primary human hepatocytes was carried out by the Department of Gastroenterology and Hepatology at the University Hospital Essen.
Primary human hepatocytes were isolated from resected liver tissue as previously described by (Zhang et al. 2012).Briefly, liver tissue was perfused and digested using two-step-collagenase-perfusion. First EGTA (0.5 mM) resolved into HBSS (Hanks balanced salt solution) and second $CaCl_2$ (5 mM)and collagenase (0.5 mg/mL) dissolved in HBSS were used for digestion to dispense the cells. Hepatocytes were enriched by centrifugation steps at 50 xg and seeded into collagen-I-coated culture plates using DMEM Ham's F12 supplemented with 10% FCS, 1% L-glutamine und 0.08 U/mL penicillin/streptomycin. Two days post preparation PHH were stimulated with 50 µg/mL poly I:C for 6 h. Total RNA was extracted and purified using the Qiazol™ and the Rneasy Mini Kit. Quantitative RT-PCR was performed with the

QuantiTect SYBR Green RT-PCR Kit using 0.1 to 0.3 µg of total RNA. Gene expression of HLA-E was determined using a comercially available primer set (Qiagen, Cat. No. QT00056567). The calculated copy numbers were normalized to β-actin, detected with forward 5´-TCCCTGGAGAAGAGCTACGA-3´ and reverse primer 5´-AGCAATGTGTTGGCGTA-CAG-3´.

3.5 Analysis of KIR genotype

KIR genotyping was carried out by the Department of for Transfusion Medicine at the University Hospital Essen.

Unlike classical human leukocyte antigens (HLA) the relevant polymorphism of Killer Cell Immunoglobulin-like receptors (KIR) is spread over the entire length of the KIR genes. Therefore, separate PCR amplifications of all exons are needed for low resolution KIR typing, which determines presence or absence of specific KIR genes in a sample. DNA of patients was extracted from PBMCs using spin columns. The KIR were genotyped using sequence-specific oligonucleotides on a Luminex™ flow nalyser platform (Heinemann 2009). In brief, this methodology consisted of a set of reverse oligonucleotide probes conjugated to fluorescently coded microspheres. Each DNA was amplified using three separate group-specific biotinylated primer sets targeting all KIR gene exons. PCR products were subsequently denatured and allowed to hybridize to complementary DNA oligonucleotide probes. Bound amplicons were tagged with nalyser din-phycoerythrin. The assignment of KIR genotypes was based on the reaction pattern determined by the Luminex™ flow nalyser using specific software (HLA Fusion, One Lambda, Inc.). Finally, all KIR genotypes were checked for consistency using all known KIR haplotypes on the Allele Frequency Net Database (www.allelefrequencies.net) (Gonzalez-Galarza et al. 2011). This database comprises approximately 300 different KIR genotypes found in more than 12,000 individuals from multiple populations. Based on the individual set of KIR genes in a sample the A KIR or B KIR haplotypes were assigned.

3.6 IFNL3 (IL-28B) and HLA class I genotyping

Genomic DNA was extracted from PBMCs using the QIAmp Blood Kit. *IL28B* rs12979860 genotyping was performed using a LightSNiP Typing Assay and the LightCycler FastStart DNA Master^{PLUS} HybProbe PCR Kit on a LightCycler 2.0

Instrument . The amplification protocol consisted of an initial denaturation step of 95 C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s and subsequent melting curve analysis. HLA-A and HLA-B typing at two-digits resolution-level was performed using sequence-specific primers methodology or alternatively using sequence-specific oligonucleotides (Oudshoorn et al. 2007).

3.7 Diagnostic methods

CMV status of 391 IDUs was analyzed using Liaison XL and the DiaSorin Liaison[TM] CMV IgG II kit.

4 Results

4.1 Patients

A total of 68 subjects were enrolled in this study, including 58 patients with a history of injection drug use (IDU) and 10 healthy controls (H). All IDUs were recruited at the Rheinische Kliniken (Clinic for addictive behavior and drug abuse) Essen. 19 anti-HCV negative (by CMIA) subjects with undetectable HCV-RNA (by RT-qPCR with a detection limit of 15 IU/ml) reporting frequent illicit intravenous drug use were considered to be exposed to HCV without getting infected (HCV seronegative IDU; SN). 19 subjects were chronically infected with HCV genotype 1, 3 or 4 (CH). Additional 20 anti-HCV positive IDUs with undetectable HCV-RNA were included and were considered as patients who had spontaneously resolved infection I. All individuals were treatment naïve. 10 anti-HCV negative healthy individuals were included as controls.

Specific host genetic markers influence HCV infection outcome. Therefore, the subjects enrolled in this study were analyzed for their *KIR2DL3* and *IFNL3* genotype. A single nucleotide polymorphism near the IFNL3 region (rs12979860) encoding interferon-λ3 was identified as a strong predictor for HCV infection outcome and treatment response in HCV genotype 1 infection. Specifically, patients with the *IFNL3* C/C genotype are more likely to resolve HCV infection than patients with the *IFNL3* C/T or T/T genotype (reviewed in (Berger et al. 2012)). Although the number of patients is not sufficient for a robust analysis, in our patient groups we did not observe a trend towards enrichment of the protective *IFNL3* genotype in the group of IDUs without HCV infection. In fact, 58% of chronically HCV infected IDUs and 58% of IDUs with resolved infection displayed the protective *IFNL3* C/C genotype while only 40% of seronegative IDUs had the protective genotype. Similar to *IFNL3*, homozygosity for the NK cell receptor *KIR2DL3* gene in combination with homozygosity for its ligand *HLA-C1* group alleles has been associated with favorable HCV infection outcome (Khakoo et al. 2004) and resistance to HCV infection (Knapp et al. 2010). Strikingly, 74 % of seronegative IDUs enrolled in this study were *KIR2DL3* homozygous while only 44-45% were *KIR2DL3* homozygous in the other

groups. Although again the cohort size is underpowered for a robust analysis, in line with previous data we observed enrichment of the favorable KIR/KIR-ligand genotype in HCV seronegative IDUs.

As risk behavior is also associated with the likelihood to acquire HCV infection (Pouget et al. 2012), we included the median duration of injection drug use in the patient characteristics. While seronegative IDUs and IDUs with resolved HCV infection reported similar median durations of intravenous drug use (R: 8.6 (1-25) years; SN: 6.7 (2-14) years), we noted a longer history of injecting drugs in chronically HCV infected patients (CH: 15.3 (2-39) years). Patient group characteristics are summarized in table 4.1.

Table 4.1: Study subjects

	Chronics	Resolved	Seronegative	Healthy
n	19	20	19	10
Age (mean)	37	36	32	32
% male	74	90	74	40
% *KIR2DL3* homozygous	44	45	74	45
% *IL28B* C/C genotype	58	60	40	n.d.
median duration of IDU	15.3 years (2-39)	8.6 years (1-25)	6.7 years (2-14)	0

n.d. not done

4.2 Phenotypic characterization of NK cells of chronically HCV infected IDUs and seronegative IDUs

NK cells are a major player in innate immunity and early immune responses in viral infections. In HCV infection distinct NK cell phenotypes have been repeatedly associated with specific outcome. We therefore decided to comparatively analyze NK cells of seronegative IDUs and chronically HCV infected IDUs to characterize a potentially protective NK cell phenotype in seronegative IDUs. NK cells are defined as $CD3^-$ $CD14^-$ and $CD19^-$ lymphocytes and can be further subdivided based on the expression of CD16 and CD56. By flow cytometric analysis we identified lymphocytes (Figure 4.1A) and excluded dead cells (Figure 4.1B). In the next step T

cells (CD3), monocytes (CD14), and B cells (CD19) (Figure 4.1C) and subsequently doublets were excluded (Figure 4.1D). The remaining cells were analyzed for the expression of CD56 and CD16 and subdivided into $CD56^{bright}CD16^-$, $CD56^{dim}CD16^+$ and $CD56^{neg}CD16^-$ NK cells (Figure 4.1E).
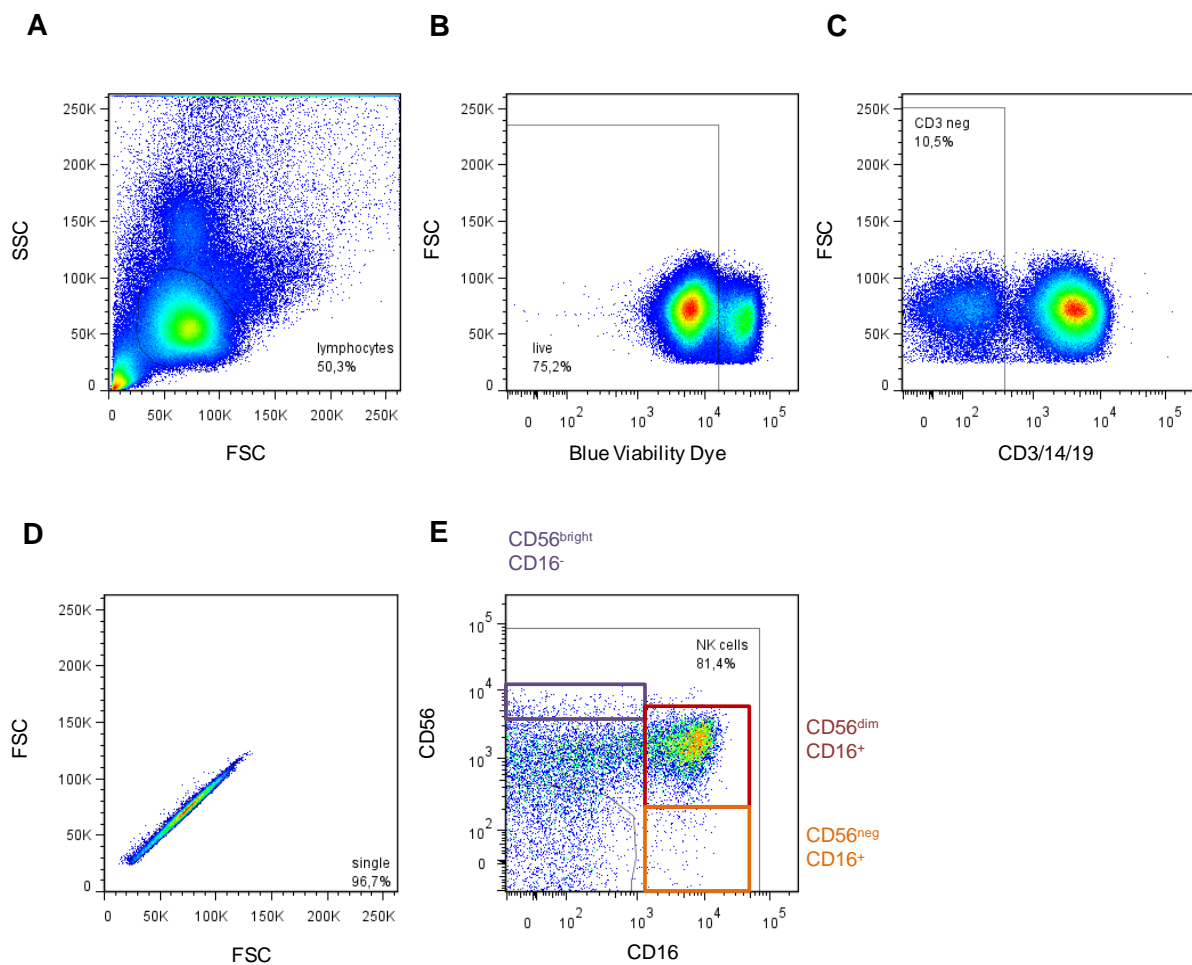

Figure 4.1: Gating strategy.
To characterize NK cells using flow cytometry, (A) lymphocytes were identified and (B) dead cells excluded. (C) In the next step T cells (CD3), monocytes (CD14) and B cells (CD19) were gated out. (D) Subsequently single cells were identified and analyzed for the expression of CD56 and CD16 and subdivided into $CD56^{bright}CD16^+$ (purple), $CD56^{dim}CD16^+$ (red) or $CD56^{neg}CD16^+$ NK cells (orange).

We analyzed the expression of a broad panel of NK cell receptors including members of to the C-type lectin like receptor family (NKG2A, NKG2C, NKG2D), the natural cytotoxicity receptor family (NKp30, NKp46) and the Killer cell Ig-like (KIR) receptor family (KIR2DL2, KIR2DS1, KIR3DL1, KIR2DL3, KIR2DL1) on NK cells of 20 seronegative IDUs and 10 chronically HCV infected IDUs. All receptor analyses were

performed on CD56^{bright}CD16$^-$ and CD56^{dim}CD16$^+$ NK cells, apart from the KIR receptors as those are solely expressed on CD56^{dim}CD16$^+$ NK cells.

Out of the eleven receptors tested NKG2C (uncorrected p=0.0236), NKp46 (uncorrected p=0.0328) and KIR2DL3 (uncorrected p=0.0426) were expressed in significantly higher frequencies on CD56^{dim} NK cells of seronegative IDUs than CD56^{dim} NK cells from chronically infected IDUs (Figure 4.2). In addition, significantly decreased numbers of NKG2A$^+$ CD56^{bright} NK cells (uncorrected p<0.0001; Figure 4.2) were observed in seronegative IDUs compared to chronically infected IDUs. Interestingly, the same trend was observed in CD56^{dim} NK cells.

4.3 Functional analysis of NK cells of chronically HCV infected IDUs and seronegative IDUs

NK cells are regulated by a complex network of activating and inhibiting receptors. To analyze functional characteristics of NK cells of patients with different HCV infection status, three different NK cell target cell lines were used mediating NK cell activation or inhibition through different receptor ligand interactions. The majority of KIR receptors inhibit NK cell activation after binding to HLA molecules. To facilitate NK cell activation all target cell lines are HLA negative. K562 cells are frequently used to activate NK cells. They mediate activation via the activating NK cell receptor NKG2D by expressing its ligands ULBP-1/2 and MIC-A/B on their surface (Bae et al. 2012). For activation of NK cells by the natural cytotoxicity receptors NKp44 and NKp46, 221 cells were used as NKp44 and NKp46 dependent lysis has been reported for this cell line (Pessino et al. 1998; Cantoni et al. 1999; Sivori et al. 1999). NK cells are also known to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) via the FcγRIII (CD16) receptor. This was mimicked by using antibody coated p815 cells as NK cell target cells. An overview of the used target cells and the engaged receptors are given in Figure 4.3.
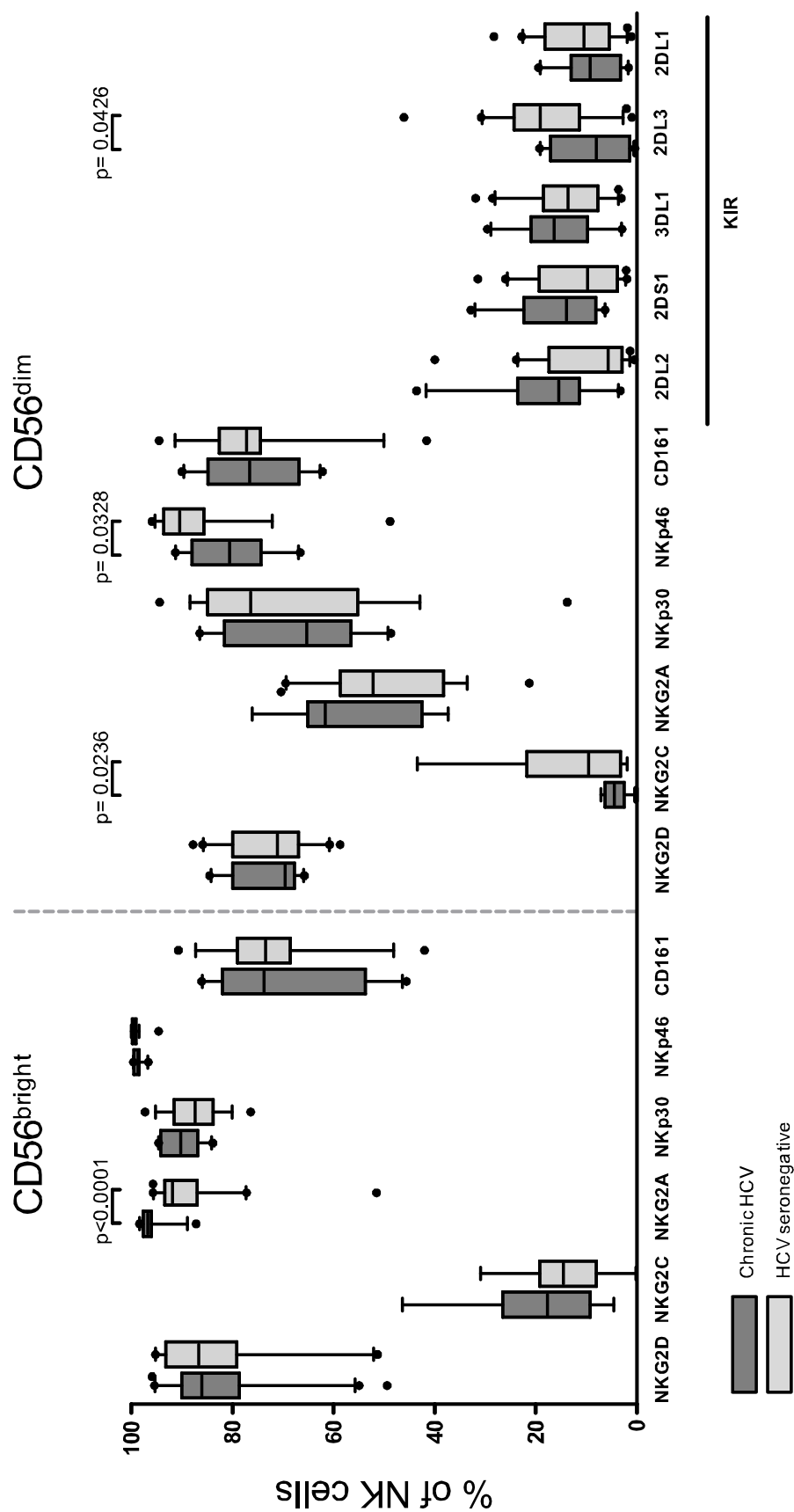
Figure 4.2: Comparative analysis of NK cells from chronically HCV infected and seronegative IDUs.
Expression of NK cell receptors was analyzed by flow cytometry on CD56bright (left panel) and CD56dim NK cells (right panel). Seronegative IDUs have lower frequencies of CD56brightNKG2A+ NK cells and higher frequencies of CD56dimNKp46+ NK cells, CD56dimNKG2C+ NK cells and CD56dimKIR2DL3+ NK cells compared to chronically infected IDUs. Non-parametric Mann-Whitney tests were used in this initial screen to compare expression frequencies of NK cell receptors with uncorrected p-values.
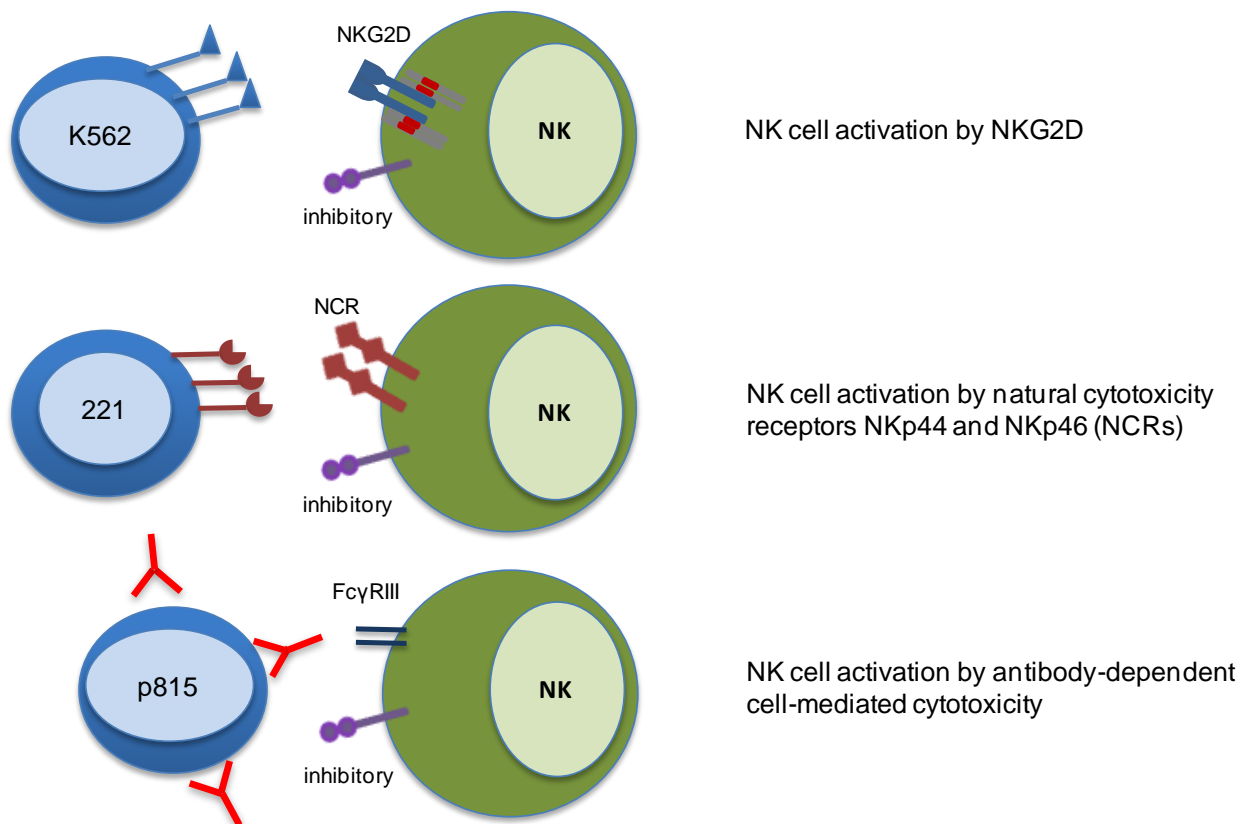
Figure 4.3: Overview of used NK cell target cell lines.
K562 cells activate NK cells by ligation of the activating NKG2A NK cell receptor. 221 cells activate NK cells by binding to NKp44 and NKp46. Antibody coated p815 cells activate NK cells FCγRIII (CD16) and are used to analyze antibody-dependent cell-mediated cytotoxicity.

Expression of the degranulation and cytotoxicity marker CD107a (Alter et al. 2004), the cytokines IFN-γ and TNF-α and the chemoattractant MIP-1β was analyzed in NK cells of 20 seronegative IDUs and 10 chronically HCV infected IDUs in response to stimulation with K562 cells, 221 cells and antibody coated p815 cells.

In line with the previously observed increased levels of NKp46^{pos} NK cells in seronegative IDUs (Figure 4.2), we observed increased CD107a production of NK cells of seronegative IDUs in response to 221 cells (uncorrected p = 0.0165, Figure 4.4). However, we could not observe differences in IFN-γ, TNF-α or MIP-1β production in response to 221 stimulation or other stimulations (Figure 4.4).
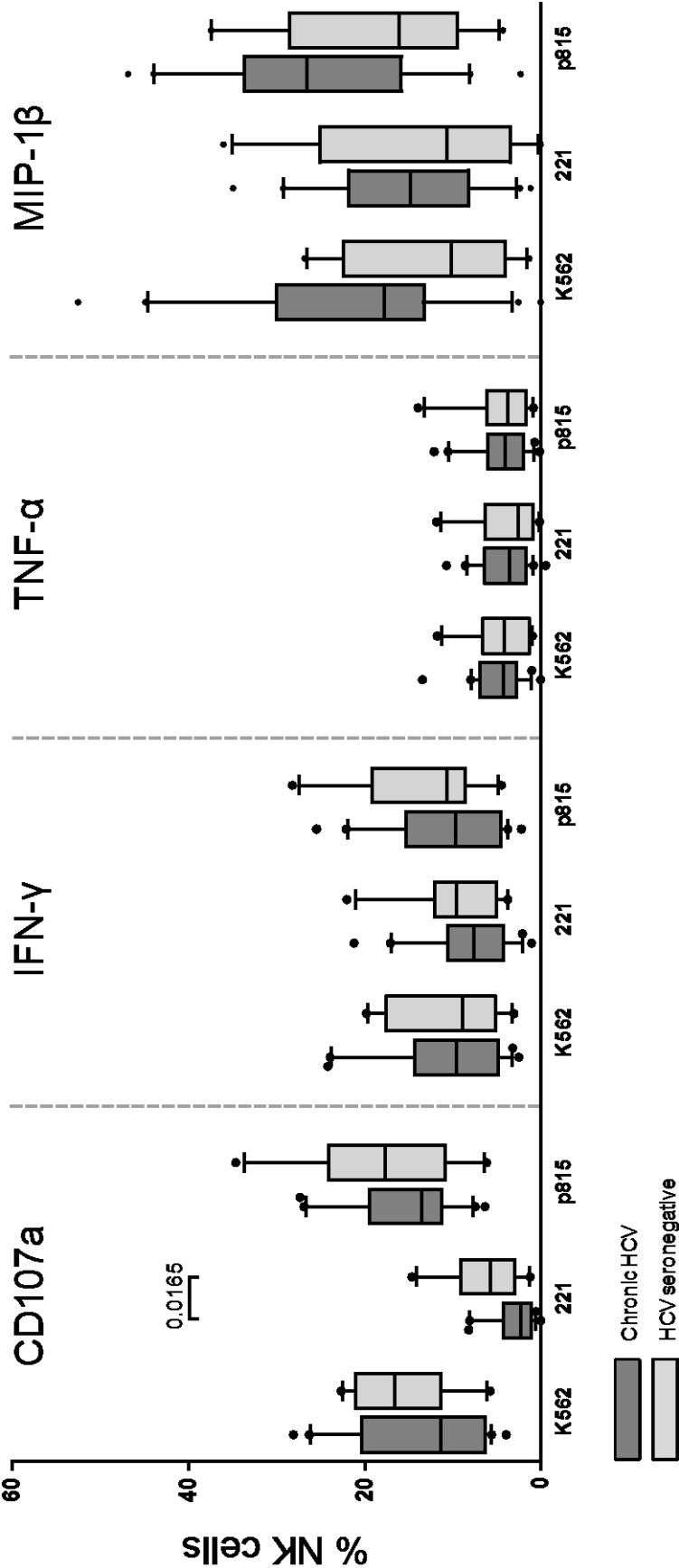
Figure 4.4: Functional analysis of NK cells from chronically HCV infected and seronegative IDUs.
NK cells of 20 seronegative and 10 chronically HCV infected IDUs were stimulated with K562 cells, 221 cells or antibody coated p815 cells and analyzed for the expression of CD107a, IFN-γ, TNF-α and MIP-1β. NK cells of seronegative IDUs produced significantly more CD107a in response to stimulation with 221 cells than NK cells of chronically HCV infected IDUs (uncorrected p= 0.0165). Non-parametric Mann-Whitney tests were used in this initial screen to compare expression of CD107a, IFN-γ, TNF-α and MIP-1β with uncorrected p-values.

4.4 Seronegative IDUs are characterized by increased frequencies of KIR2DL3$^+$NKG2A$^-$ NK cells

As we observed a very pronounced difference in NKG2A expression between HCV seronegative IDUs and chronically infected IDUs and *KIR2DL3* genotype has repeatedly been associated with HCV susceptibility and resolution (Khakoo et al. 2004; Knapp et al. 2010), we decided to characterize NKG2A and KIR2DL3 expression in IDUs in greater detail. For that purpose, NK cells of nineteen IDUs with chronic HCV infection (CH), twenty IDUs with resolved HCV infection I, nineteen seronegative (SN) IDUs and ten healthy controls (H) were identified and simultaneously stained for NKG2A and KIR2DL3. Individuals lacking the KIR2DL3 gene were excluded from the analysis. A representative staining and the gating strategy are given in Figure 4.5. As KIR2DL3 is almost exclusively expressed on CD56^{dim} NK cells while NKG2A is expressed on up to 100% of CD56^{bright} NK cells, CD56^{dim} NK cells were used for the analysis.
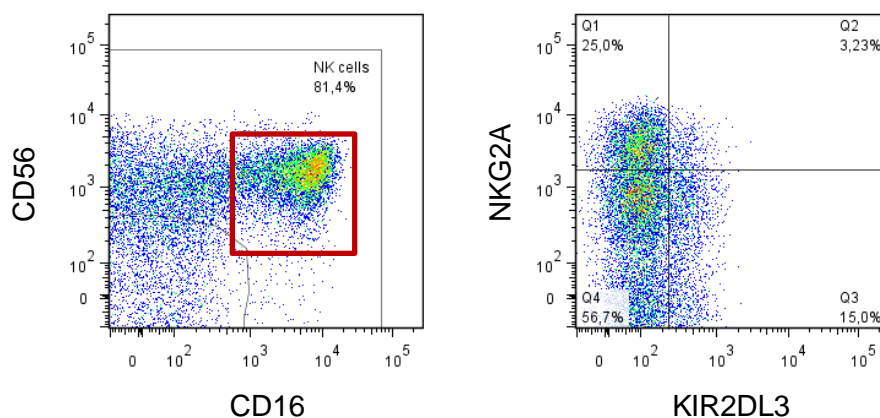

Figure 4.5: Gating strategy.
NK cells were characterized as described above and KIR2DL3$^-$NKG2A$^+$, KIR2DL3$^+$NKG2A$^+$, KIR2DL3$^+$NKG2A$^-$ and KIR2DL3$^-$NKG2A$^-$ NK cells were identified.

HCV seronegative IDUs had significantly increased frequencies of CD56^{dim}KIR2DL3$^+$NKG2A$^-$ NK cells (median: 16.8%) compared to IDUs with chronic HCV infection (median: 6.47%; p<0.0001), healthy controls (median 4.65%; p<0.001) and IDUs with resolved HCV infection (median 8.67; p<0.05; Figure 4.6A). In turn, analysis of CD56^{dim}KIR2DL3$^-$NKG2A$^+$ NK cells revealed decreased frequencies in

HCV seronegative IDUs (median: 27%) compared to healthy controls (median: 52.1; p<0.001) and IDUs with chronic HCV infection (median: 48.5%; p<0.001; Figure 4.6B). Interestingly, IDUs with resolved HCV infection had intermediate frequencies ranging between HCV seronegative IDUs and IDUs with chronic HCV infection (median 37.1%; Figure 4.6B). The combination of increased levels of KIR2DL3$^+$NKG2A$^-$ NK cells and decreased levels of KIR2DL3$^-$NKG2A$^+$ NK cells in seronegative IDUs suggested a negative correlation between KIR2DL3 and NKG2A expression. Indeed, an analysis of CD56^{dim} NK cells of all patients revealed a strong inverse correlation between the frequency of KIR2DL3$^+$ and NKG2A$^+$ NK cells (p<0.0001, r=-0.5341; Figure 4.6C).

4.5 NKG2C expression correlates with NKG2A and KIR2DL3 expression

NKG2A and NKG2C are both members of the C-type lectine like NK cell receptor familiy, forming heterodimers with CD94 on the cell surface. Both receptors bind to the same lignd, HLA-E, with different affinities and mediate different signals to NK cells. NKG2A binds HLA-E with higher affinity than NKG2C and mediates inhibiting signals to NK cells, while NKG2C binds HLA-E with low affinity and is an activating NK cell receptor (Aldrich et al. 1994; Borrego et al. 1998). As it has previoulsy been reported that NK cells expressing high levels of NKG2A express low levels of NKG2C and vice versa (Beziat et al. 2012), we analyzed NK cells of IDUs with different HCV infection status and healthy controls for NKG2C expression.

An analysis including all patients independent of HCV infection status revealed an inverse correlation between NKG2A and NKG2C expression on CD56^{dim} NK cells (p= 0.0056; r= -0.3926; Figure 4.7A). Interestingly, there was also a weak but statistically significant negative correlation bewteen NKG2C and KIR2DL3 expression (p= 0.0047; r=0.2179; Figure 4.7B). We did not observe significant differences in NKG2C expression in the different study groups, however a strong tendency towards increased frequencies of NKG2C$^+$ NK cells in seronegtive IDUs and IDUs with resolved HCV infection was observable (Figure 4.7C).
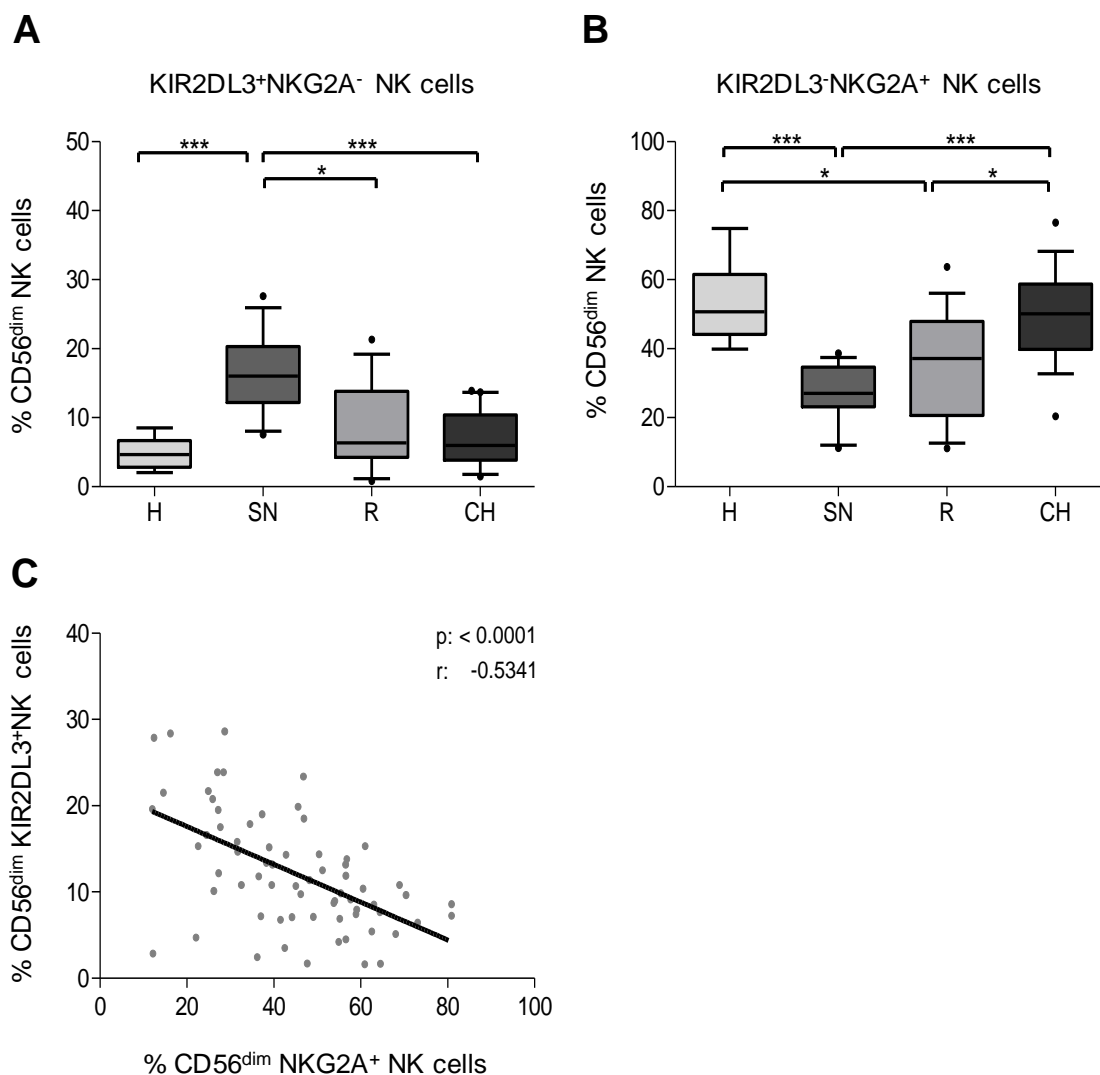
Figure 4.6: HCV seronegative IDUs have increased frequencies of KIR2DL3$^+$NKG2A$^-$ CD56^{dim} NK cells.
NK cells of healthy controls (H), seronegative IDUs (SN), IDUs with resolved HCV infection I and chronically HCV infected IDUs (CH) were analyzed. (A) SN had significantly higher frequencies of CD56^{dim}KIR2DL3$^+$NKG2A$^-$ NK cells compared to H, CH and R. (B) SN had significantly lower frequencies of CD56^{dim}KIR2DL3$^-$NKG2A$^+$ NK cells compared to H and CH (Kruskal-Wallis; ***p<0.001; *p<0.05). (C) In addition there was an inverse correlation between the frequency of CD56^{dim}KIR2DL3$^+$ and CD56^{dim}NKG2A$^+$ NK cells (p<0.0001; r=-0.5341).
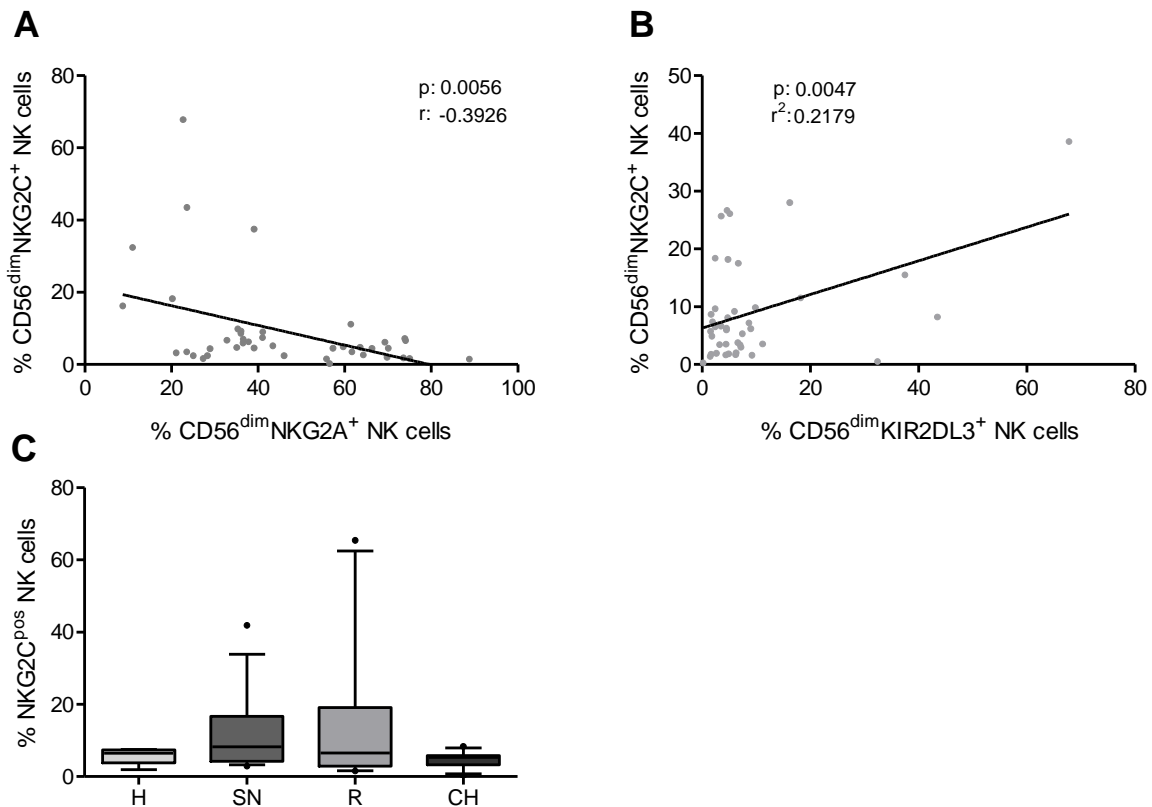
Figure 4.7: NKG2C expression correlates with NKG2A und KIR2DL3 expression.
NKG2C, NKG2A and KIR2DL3 expression on CD56^{dim} NK cells of IDUs with different HCV infection outcome were analyzed. (A) NKG2C and NKG2A expression was inversely correlated correlated (p= 0.0056; r= -0.3926). (B) NKG2C and KIR2DL3 expression was also correlated (p= 0.0047; r=0.2179). (C) A strong trend towards increased levels of NKG2C$^+$ NK cells in seronegative IDUs and IDUs with resolved HCV infection was observed, however, the differences were not statistically significant.

4.6   NK cell subset distribution and CD57 expression do not vary in between patients

As KIR2DL3 and NKG2C are predominatly expressed on CD56^{dim} NK cells and NKG2A is predominatly expressed on CD56^{bright} NK cells, we analyzed the NK cell subset distribution among patients with different HCV infection outcome to explore the possibility that the KIR2DL3$^+$NKG2A$^-$ NK cell phenotype observed in seroneagtive IDUs was a result of a different NK cell subset distribution. However, the analysis revealed that there was no difference in subset distribution in between patient groups (Figure 4.8A). To further define the maturation status, NK cells were analyzed for the expression of CD57. CD57 is reported to be expressed on previouly activated NK cells (Bjorkstrom et al. 2010; Lopez-Verges et al. 2010) and follows a specific expression pattern in CD56^{bright}, CD56^{dim} and CD56^{neg} NK cells. In our analysis CD57

47

followed the pattern expected for the different NK cell subsets, however we could not observe a difference in CD57 expression between the different patient groups (Figure 4.8B).
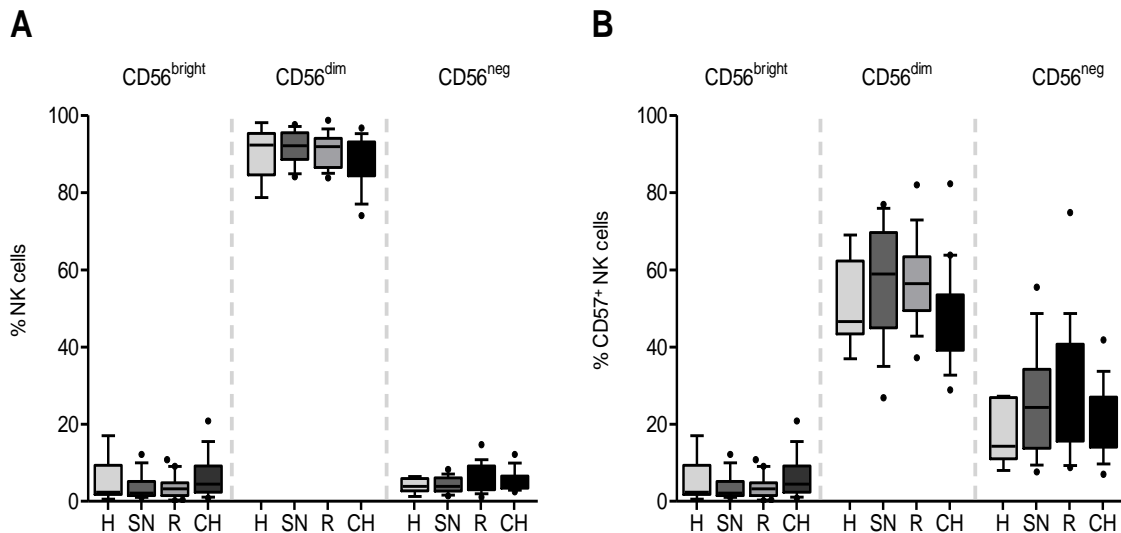

Figure 4.8: CD57 expression and subset distribution.
NK cells of healthy controls (H), seronegative IDUs (SN), IDUs with resolved HCV infection I and chronically HCV infected IDUs (CH) were characterized and subset distribution and CD57 expression was analyzed. There was no difference in (A) subset distribution of CD56bright, CD56dim and CD56neg NK cells and (B) CD57 expression between patient groups.

4.7 The NKG2A/NKG2C ligand HLA-E is upregulated in HCV infected livers

Previous reports suggest a protective effect of homozygosity for *KIR2DL3* and HLA-C1 in HCV infection. However, a direct mechanism explaining conclusively how KIR2DL3 protects from HCV infection has never been described. In fact, it is counterintuitive that an inhibiting NK cell receptor is protective in HCV infection. Here we hypothesize that the protective effect is rather mediated by the absence of NKG2A than the presence of KIR2DL3. To further strengthen this hypothesis, we analyzed the intrahepatic expression of HLA-E, the ligand for the inhibiting NK cell receptor NKG2A and the activating NK cell receptor NKG2C.

In cooperation with the Department of Gastroenterology and Hepatology at the University Hospital Essen, HLA-E expression profiles derived from liver biopsies were compared between 51 patients with HCV infection and 22 patients with Hepatitis B virus (HBV) infection. Expression of HLA-E was significantly higher in HCV infected

patients compared to HBV infected patients (p=0.002, Figure 4.9A). Interestingly, the data also revealed a positive correlation between HCV viral load and intrahepatic HLA-E transcript levels (p=0.0029; r= 0.3223; Figure 4.9B), while a similar analysis with HBV patients revealed no correlation (data not shown). Unlike HBV, HCV induces a strong type I interferon response in the infected liver which can be simulated by treatment with the TLR3 agonist poly I:C *in vitro*. Therefore, primary human hepatocytes were treated with 50 µl/ml poly I:C for 6 h and the HLA-E transcript level was determined by quantitative PCR. The results support that HLA-E mRNA levels are upregulated in response to poly I:C stimulation. On average a 2.9-fold increase in HLA-E transcript levels was observed after poly I:C stimulation (p<0.0001; Figure 4.9C).
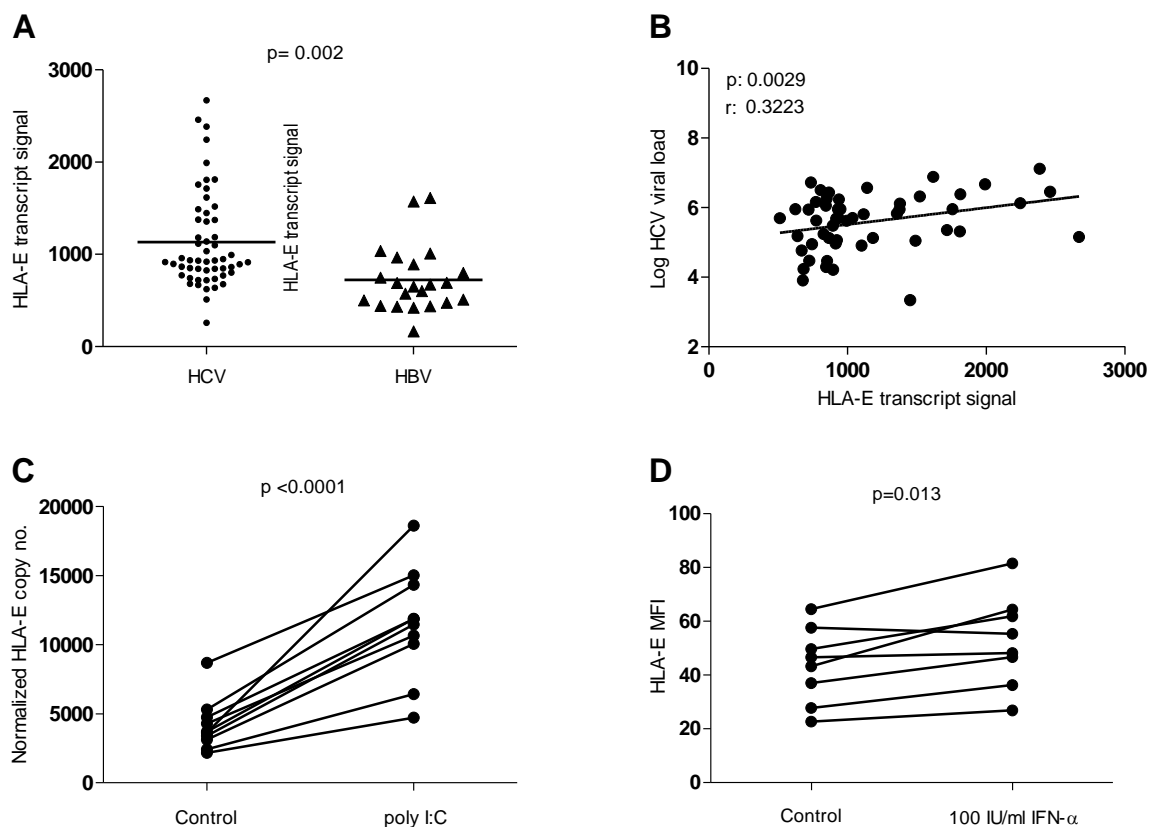

Figure 4.9 The NKG2A ligand HLA-E is upregulated in HCV infected livers.

(4) HLA-E transcript levels were determined in liver biopsies from patients with HCV infection and patients with HBV infection. HCV infected patients showed significantly higher HLA-E transcript signals compared to HBV infected patients (Mann-Whitney; p=0.002). (B) HCV viral load correlated with the HLA-E transcript signals (p=0.002; r= 0.3223). (C) PHHs were stimulated with 50 µl/ml poly I:C or medium for 6 h. After treatment HLA-E RNA copy numbers were assessed by quantitative PCR. Poly I:C treated PHHs had significantly increased HLA-E RNA copy numbers compared to medium treated controls (paired t-test; p<0.0001). (D) PHHs were treated with 100 IU/ml IFN-α or medium alone. After 24h HLA-E surface expression was analyzed using flow cytometry. IFN-α treated PHHs had significantly increased HLA-E levels (MFI) compared to untreated PHHs (paired t-test; p=0.013).

Taken together, there is strong evidence that HLA-E transcription is elevated in the liver upon infection with HCV. However, it is unclear if higher transcript levels result in higher HLA-E expression levels of the protein on the cell surface, because HLA-E needs to be stabilized by binding of leader peptide derived from other HLA molecules. We therefore addressed if treatment with IFN-α directly affects HLA-E protein expression at the cell surface of primary human hepatocytes (PHH). HLA-E expression was analyzed by flow cytometry on PHH with and without prior IFN-α treatment. Compared to the untreated control there was a significant upregulation of HLA-E on the cell surface of PHH in the presence of IFN-α (p=0.013; Figure 4.9D).

4.8 Induction of HLA-E expression on K562 HLA-E cells

The previously described NK cell target cell lines do not express HLA-E and are thus not suited to analyze NK cell functionality in the context of HCV induced HLA-E expression. We therefore analyzed HLA-E expression on K562 cells stably transfected with *HLA-E*01033* (K562 HLA-E cells). As Nattermann et al. previously described stabilization of HLA-E by external application of the HCV core$_{35-44}$ peptide (Nattermann et al. 2005), we analyzed HLA-E expression on normal K562 cells and K562 HLA-E cells in the presence or absence of core$_{35-44}$. In the presence of core$_{35-44}$, K562 HLA-E cells expressed high levels of HLA-E while only intermediate levels were expressed in the absence of the peptide (Figure 4.10A). K562 HLA-E cells were loaded with the core$_{35-44}$ peptide at 28 °C over night. Assays assessing NK cell functionality are best carried out at 37 °C for a period of 5 h. We therefore tested the stability of HLA-E expression on K562 HLA-E cells at 37 °C over 5 h. While the cells expressed high levels of HLA-E directly after they were loaded with peptide at 28 °C, HLA-E expression rapidly declined at 37 °C. After one hour HLA-E expression was significantly reduced and after 2 h HLA-E expression was already barely detectable (Figure 4.10B). As K562 cells alone already expressed increased HLA-E levels and peptide induced HLA-E expression was instable at 37 °C, unloaded K562 HLA-E cells and K562 cells were used for the following experiments.
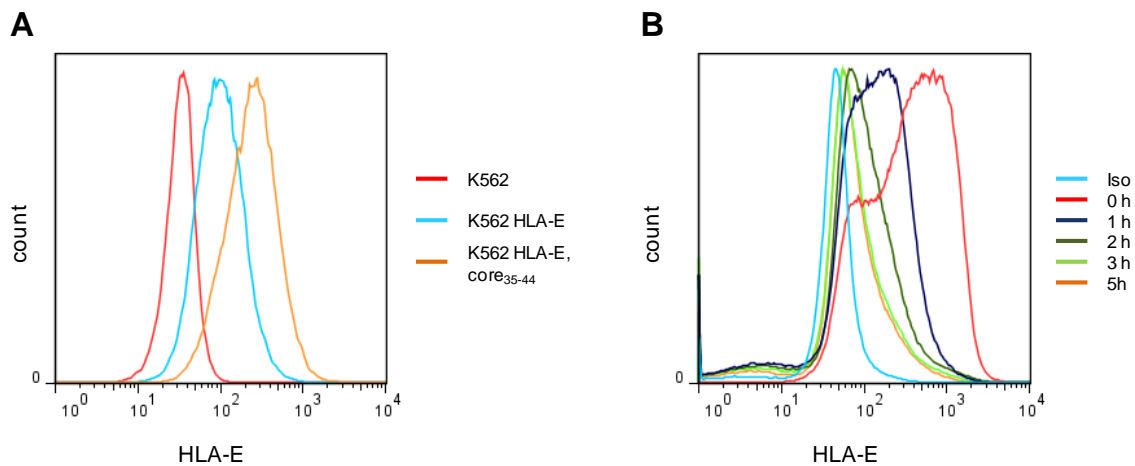
Figure 4.10: HLA-E expression by K562 and K562 HLA-E cells.
HLA-E expression of K562 and K562 HLA-E cells was analyzed in the presence and absence of $core_{35-44}$ peptide. (A) K562 HLA-E cells loaded with the $core_{35-44}$ peptide displayed high levels of HLA-E on the cell surface while K562 HLA-E NK cells displayed intermediate HLA-E expression levels. (B) K562 HLA-E cells were loaded with $core_{35-44}$ peptide at 28 °C over night and subsequently transferred to 37 °C. HLA-E expression decreased signifivantly after 1 h at 37 °C and was hardly detectable after 2 h.

4.9 NKG2A enriched NK cells show a trend towards decreased degranulation in the presence of HLA-E

As NK cells in seronegative IDUs are predominantly $KIR2DL3^+NKG2A^-$ while NK cells in chronically HCV infected IDUs are predominately $KIR2DL3^-NKG2A^+$, NKG2A enriched and NKGA depleted NK cells of ten healthy individuals were comparatively analyzed. For that purpose, NK cells were enriched using an NK cell isolation kit allowing the enrichment of untouched NK cells. Subsequently, the NK cell enriched cell suspension was stained with a PE labeled NKG2A specific antibody and subdivided into an NKG2A enriched NK cell fraction and an NKG2A depleted NK cell fraction using a PE selection kit. For simplification the NKG2A enriched fraction will be referred to as the $NKG2A^{pos}$ NK cell fraction and the NKG2A depleted fraction will be referred to as the $NKG2A^{neg}$ NK cell fraction. As HLA-E expression is upregulated in the HCV infected liver, we stimulated the $NKG2A^{pos}$ NK cell fraction and the $NKG2A^{neg}$ NK cell fraction with K562 cells (HLA-E low) or K562 HLA-E cells (HLA-E high) (Nattermann et al. 2005). After 5 hours of stimulation CD107a expression was analyzed to assess degranulation. However we did not observe inhibition of NKG2Apos or NKG2Aneg NK cells in the presence of HLA-E (Figure 4.11A, B).
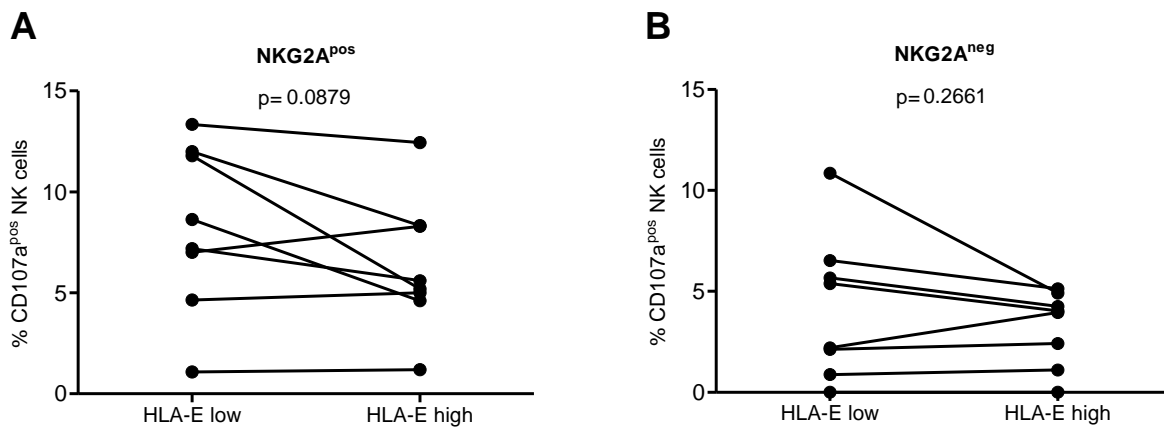
Figure 4.11: Functional analysis of NKG2A enriched and depleted NK cells.
NK cells were enriched or depleted for NKG2A and stimulated with HLA-E high (K562 HLA-E) or HLA-E low expressing (K562) NK call target cells. (A) The NKG2A enriched fraction showed a trend towards inhibition in the presence of HLA-E high target cells (B) while NKG2A depleted NK cells were not inhibited in the presence of HLA-E. Wilcoxon matched pairs tests were used to compare groups and resulting p-values are indicated.

4.10 HLA-E inhibits IFN-γ and CD107a production by KIR2DL3⁻NKG2A⁺ NK cells but not KIR2DL3⁺NKG2A⁻ NK cells

As NK cells might need the support of accesory cells to develop their full functional capacity, whole PBMCs from 10 healthy donors were stimulated with normal K562 cells (HLA-E low) or K562 HLA-E cells (HLA-E high). IFN-γ production was significantly decreased upon stimulation with HLA-E expressing K562 cells compared to stimulation with native K562 cells in total NK cells (p= 0.0039; Figure 4.12A). As KIR2DL3⁺NKG2A⁻ NK cells represent NK cells typically found in HCV seronegative IDUs and KIR2DL3⁻NKG2A⁺ NK cells represent NK cells typically found in chroncally HCV infected IDUs, those NK cell subtypes were further analysed using flow cytometry. KIR2DL3⁻NKG2A⁺ NK cells were efficiently inhibited in the presence of HLA-E expressing target cells (p=0.0039; Figure 4.12B) consistent with an inhibitory signal conferred by HLA-E. In contrast, no significant inhibition of IFN-γ production was observed in the KIR2DL3⁺NKG2A⁻ NK cell subset (Figure 4.12C), which is the NK cell phenotype predominantly observed in HCV seronegative IDUs. The same results were obtained when the degranulation marker CD107a was analyzed in the different NK cell subsets in the absence and presence of HLA-E (Figure 4.12D-E).
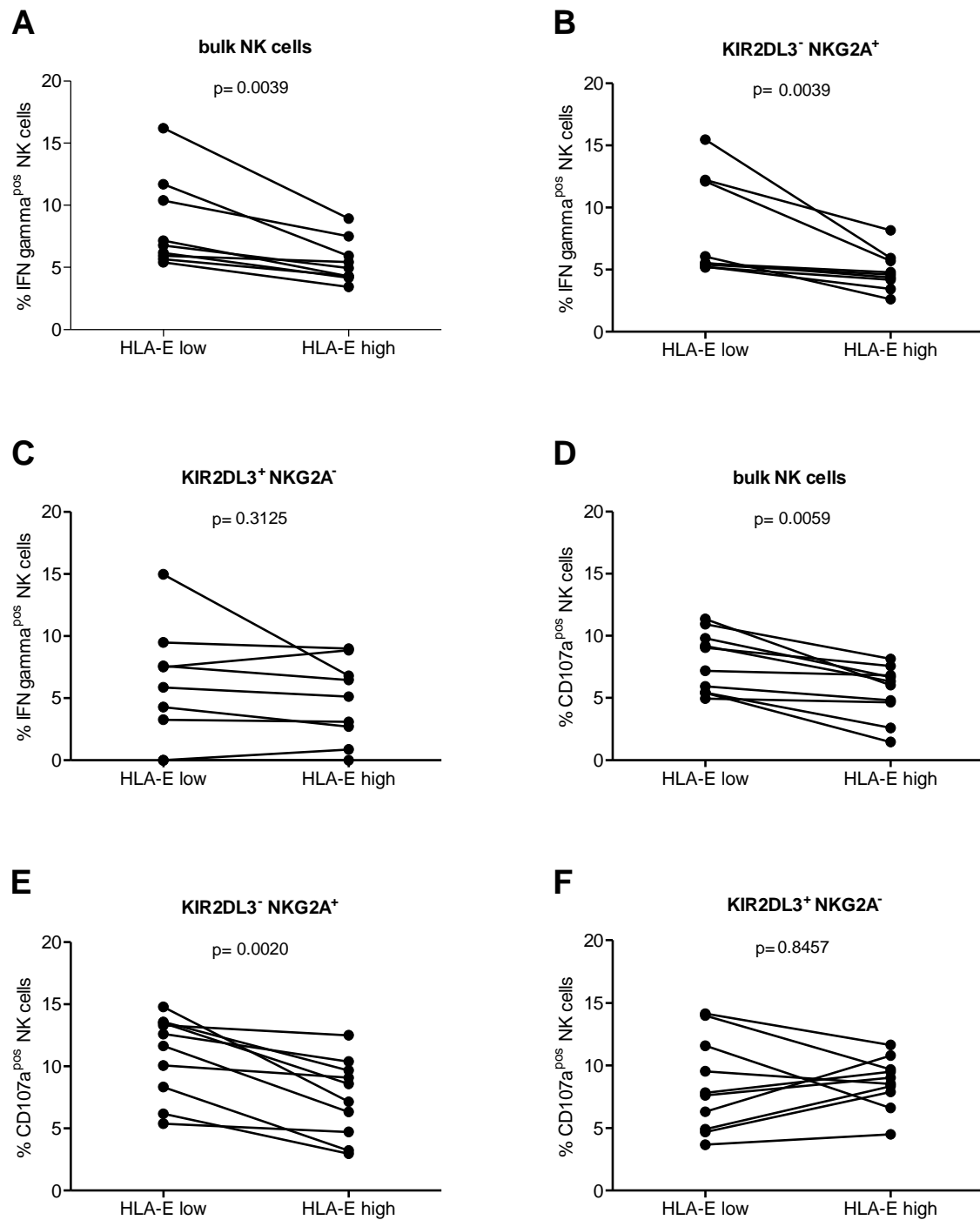
Figure 4.12: KIR2DL3+NKG2A- NK cells are inhibited in the presence of HLA-E.
PBMCs of healthy individuals were stimulated with HLA-E low or high expressing NK cell target cells and CD107a and IFN-γ production by different NK cell subtypes was analyzed. (A) IFN-γ production by bulk NK cells and (B) KIR2DL3-NKG2A+ NK cells is inhibited in the presence of HLA-E. (C) IFN-γ production by KIR2DL3+NKG2A- NK cells was not inhibited in the presence of HLA-E. (D-E) Similar results were obtained, when CD107a expression was analyzed. Wilcoxon matched pairs tests were used to compare groups and resulting p-values are indicated.

4.11 HLA-E inhibits IFN-γ production by NK cells of chronically HCV-infected IDUs but not by NK cells of seronegative IDUs

As KIR2DL3$^+$NKG2A$^-$ NK cells of healthy individuals were not inhibited in the presence of HLA-E, while KIR2DL3$^-$NKG2A$^+$ NK cells were potently inhibited, we next tested fuctionality of NK cells of seronegative IDUs and chronically HCV infeceted IDUs in the presence and absence of HLA-E directly. For that purpose PBMCs of ten chronically HCV infected IDUs and 10 seronegative IDUs were incubated with K562 and K562 HLA-E NK cell target cells at an effetctor:target ratio of 10:1 and IFN-γ productions of NK cells was analyzed using flow cytometry. IFN-γ production by NK cells from chonically HCV infected IDUs was significantly inhibited in the presence of HLA-E (p=0.0017, Figure 4.13A), while there was no inhibitory effect on IFN-γ production by NK cells from HCV seronegative IDUs (Figure 4.13B).
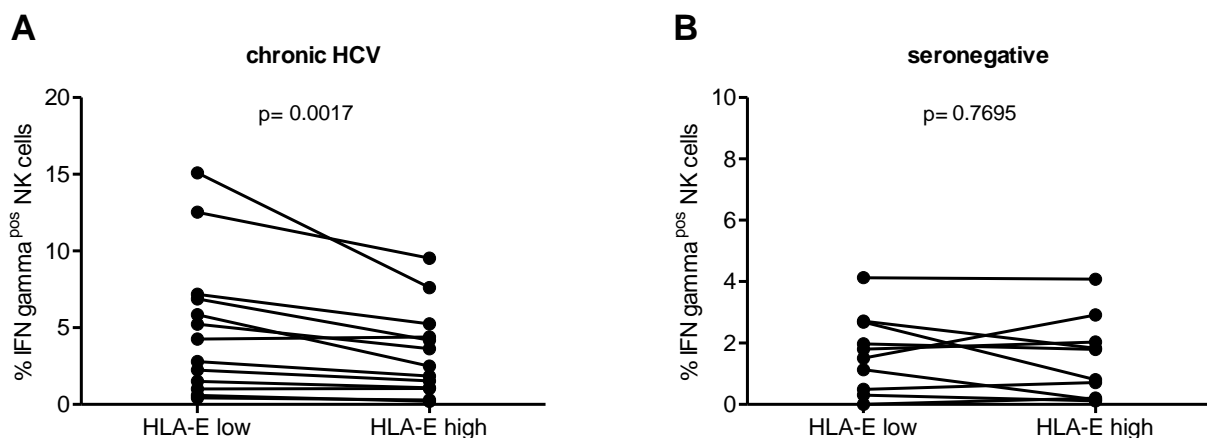
Figure 4.13: NK cells of chronically HCV infected IDUs but not seronegative IDUs are inhibited in the presence of HLA-E.
PBMCs of chronically HCV infected and seronegative IDUs were stimulated with HLA-E high and low expressing NK cell target cell lines. (A) NK cells of chronically HCV infected IDUs were inhibited in the presence of HLA-E (B) while NK cells of seronegative IDUs were not inhibited by HLA-E. Wilcoxon matched pairs tests were used to compare groups and resulting p-values are indicated

4.12 The Human cytomegalovirus (HCMV) serostatus has an impact on the NK cell phenotype of IDUs

Human cytomegalovirus infects all human populations with a prevalence ranging from 50 %-100 % depending on socioeconomic factors (Dowd et al. 2009) and has previously been described to leave a stable imprint in the NK cell population. Productive HCMV infection results in a persistent increase of NKG2C$^+$ NK cells displaying KIR receptors specific for HLA class I molecules (Muntasell et al. 2013).

Moreover, an expansion of KIR2DL3⁺NKG2C⁺ NK cells has been described as a result of HCMV infection (Beziat et al. 2012). Taken together, the existing data suggest that the phenotype of NK cells of HCMV seropositive individuals is reminiscent of the NK cell phenotype we describe for HCV seronegative IDUs. We therefore analyzed the NKG2A; NKG2C and KIR2DL3 expression on NK cells of anti-HCMV positive and negative IDUs.

Indeed, we observed a trend towards higher frequency of NKG2C⁺ and KIR2DL3⁺ NK cells in anti-CMV positive IDUs compared to anti-CMV negative IDUs (Figure 3.14A, B). Importantly we observed significantly decreased frequencies of NKG2A⁺ NK cells in anti-CMV positive IDUs (mean: 37.3%) compared to anti-CMV negative IDUs (mean: 51.2%; p= 0.0016; Figure 4.14C).
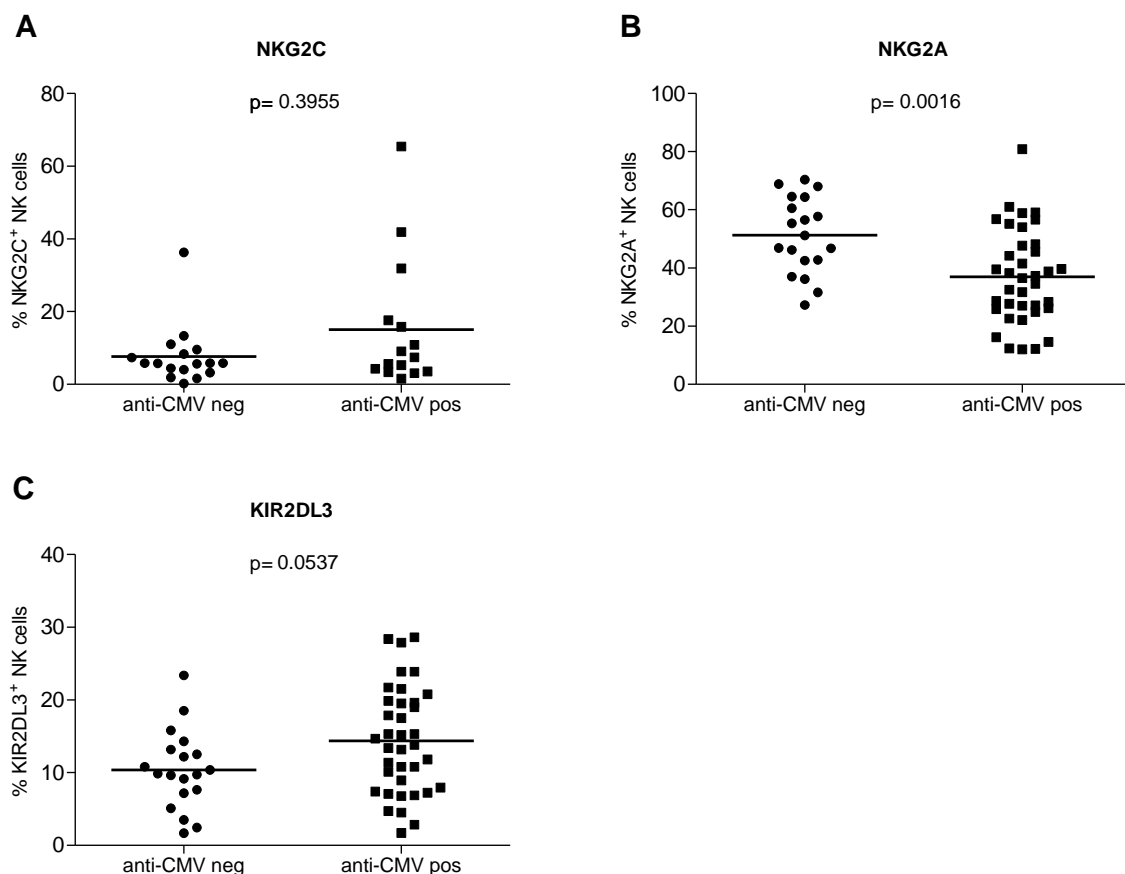
Figure 4.14: Phenotypical analysis of NK cells of anti-HCMV positive and negative IDUs.
NK cells of anti-HCMV positive and HCMV negative IDUs were analyzed for the expression of NKG2C; NKG2A and KIR2DL3. (A) Anti-HCMV positive IDUs showed a trend towards increased levels of NKG2C⁺ NK cells. (B) Frequencies of NKG2A⁺ NK cells were significantly decreased in anti-CMV positive IDUs (t-test; p=0.0016) (C) while they displayed a trend towards increased frequencies of KIR2DL3 expressing NK cells.

4.13 Human cytomegalovirus (HCMV) infection potentially influences HCV infection outcome

We hypothesized that anti-CMV positive patients are enriched in the group of IDUs with resolved HCV infection and the group of HCV seronegative IDUs. To analyze this, we tested a total of 391 IDUs, 253 chronically HCV infected, 92 with resolved HCV infection and 46 seronegative IDUs for HCVM specific Immunoglobulin G (IgG). While only 52% of chronically HCV infected IDUs were HCVM seropositive, 60% of IDUs with resolved HCV infection and 59% of HCV seronegative IDUs tested positive for HCMV specific IgG (Figure 4.15A), however, the difference was not statistically significant. As HCMV prevalence is known to increase with age (Lubeck et al. 2010) we compared the median age of 67 HCV seronegative, 189 IDUs with chronic HCV infection and 87 IDUs with resolved HCV infection,. In fact HCV seronegative IDUs were significantly younger than IDUs with chronic ($p<0.01$) or IDUs with resolved HCV infection ($p<0.01$; Figure 4.15B). The median age of seronegative IDUs was 33.8 years, in contrast, the median age of IDUs with chronic infection was 38.0 years and the median age of IDUs with resolved HCV infection was 38.3 years. The trend towards higher HCMV seroprevalence in the significantly younger group of HCV seronegative IDUs is unexpected and may in fact be biologically relevant for maintenance of the HCV seronegative state.
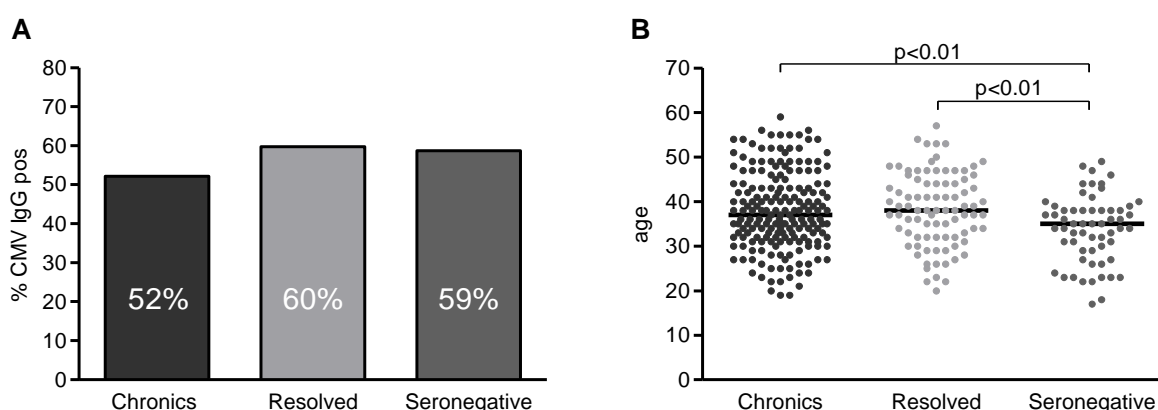
Figure 4.15: Anti-HCMV prevalence and age in IDUs with different HCV infection outcome.
52% of chronically HCV infected IDUs, 60% of IDUs with resolved HCV infection and 59% of HCV seronegative IDUs tested positive for HCMV specific IgG. (B) The age of 67 HCV seronegative IDUs, 189 chronically HCV infected IDUs and 87 IDUs with resolved HCV infection was compared. HCV seronegative IDUs are significantly younger than IDUs with chronic ($p<0.01$) of resolved infection (p0.1).

5 Discussion

5.1 NK cells in HCV infection

While several factors including *IFLN3* genotype (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009; Rauch et al. 2010) and vigorous early T cell responses (Lechner et al. 2000; Thimme et al. 2001; Urbani et al. 2002) are known to influence spontaneous resolution of HCV infection those factors do not mediate initial protection from infection. Little is known about the mechanisms resulting in resistance to HCV infection, however several reports indicate that NK cells might play a central role (Golden-Mason et al. 2010; Knapp et al. 2010; Werner et al. 2013). Here, we explored a large panel of NK cell receptors in a cohort of HCV seronegative IDUs with continuous risk behavior and compared their NK cell receptor expression profiles to IDUs with chronic HCV infection. Interestingly HCV seronegative IDUs showed higher frequencies of $KIR2DL3^+$, $NKG2C^+$ and $NKp46^+CD56^{dim}$ NK cells and lower frequencies of $NKG2A^+CD56^{bright}$ NK cells compared to IDUs with chronic HCV infection.

In line with the increased NKp46 expression in seronegtive IDUs in this inicial screen, we observed increased CD107a production by NK cells in response to 221 NK target cells, activating NK cells via NKp44 and NKp46. This data conclusively demonstrates increased NKp46 mediated fuctionality of NK cells in seroneagtive IDUs. While there is no data linking NKp46 expression to protection from HCV infection, there are reports that convincingly linked NKp46 to increased NK cell function in HCV infection. Kramer et al. identified $NKp46^{dim}$ and $NK46^{high}$ NK cells based on the density of NKp46 surface expression and coexpression of maturation markers. Importantly they were able to show that $NKp46^{high}$ NK cells displayed increased functionality in regard to cytolytic activity and IFN-γ production in general and in response to the HCV replicon system. Intrahepatic NK cells displayed the same phenotype, and the frequency of intrahepatic $NKp46^{high}$ NK cells was inversely correlated with HCV-RNA levels and fibrosis stage (Kramer et al. 2012). Taken together our data and the data by Kramer et al. indicate a beneficial effect of increased NKp46 levels in protection from HCV infection and viral control associated with increased NK cell functionality.

However, the question which ligand mediates this NKp46 mediated activation in HCV infection remains elusive. NKp46 is known to directly bind to viral antigens of influenza viruses (Mandelboim et al. 2001), however, an HCV specific ligand is unknown. Interestingly NKp44, was reported to bind to the E2 protein of other Flaviviruses (Dengue and West Nile Virus) (Hershkovitz et al. 2009), suggesting that NCR might also be able to directly bind to HCV E proteins.

A study on HCV negative high-risk IDUs by Golden-Mason et al. analyzed expression of the natural cytotoxicity receptors NKp30 and NKp44 in a prospective cohort of IDUs with subsequent HCV genotype 1a infection and patients who remained seronegative (Golden-Mason et al. 2010). The authors observed higher expression levels of NKp30 associated with increased cytotoxicity in IDUs who remained HCV seronegative, however, no analyses of other NK cell receptors were performed. In our cohort of IDUs there was a minor trend towards higher expression of NKp30 in HCV seronegative IDUs, although the difference was not statistically significant. One important difference between the two study cohorts is the HCV genotype distribution. In North America IDUs are almost exclusively infected with HCV genotype 1a (Cox et al. 2005; Rustgi 2007) whereas in Germany infections with HCV genotype 1a, 1b and 3a are common (Ross et al. 2000; Esteban et al. 2008). We therefore included in our cohort IDUs with HCV genotype 1 and 3 infections. In fact, there is strong immunological evidence that most IDUs are exposed to both genotypes (Giugliano et al. 2009). It seems possible that the protective effect of NKp30 is HCV genotype-dependent and predominantly operative against genotype 1a.

Although genetic association studies have repeatedly shown that the *KIR2DL3* and *HLA-C* genotype have an impact on the outcome of HCV infection (Khakoo et al. 2004; Knapp et al. 2010), to our knowledge this is the first study reporting increased protein expression of KIR2DL3 on NK cells of seronegative IDUs and IDUs with resolved infection. Of note, in line with these previous genetic association studies we also observed a higher frequency of HCV seronegative IDUs homozygous for *KIR2DL3* compared to IDUs with chronic HCV infection (74% HCV seronegative IDUs versus 44% IDUs with chronic infection). Previous attempts to explain the

protective effect of *KIR2DL3* and *HLA-C1* homozygosity in viral infection argued towards a weaker interaction between KIR2DL3 and HLA-C1 compared to KIR2DL1 HLA-C2 interaction (Hiby et al. 2004; Khakoo et al. 2004; Parham 2004), resulting in less KIR2DL3 mediated inhibition and easier activation of KIR2DL3^{+} NK cells Consistent with this notion, Winter et al. showed weaker binding between KIR2DL3-Fc fusion proteins and HLA-C1 transfectants compared to KIR2DL1-Fc fusion proteins and HLA-C2 transfectants (Winter et al. 1998). However, direct measurements by surface plasmon resonance revealed almost similar affinity between KIR2DL3 and HLA-C1 and KIR2DL1 and HLA-C2, respectively (Vales-Gomez et al. 1998; Maenaka et al. 1999). A study by Ahlenstiel et at. Described earlier and increased production of CD107a and IFN-γ by NK cells of *KIR2DL3 HLA-C1* homozygous patients in response to influenza A virus infected monocytes (Ahlenstiel et al. 2008), demonstrating increased antiviral functions of NK cells of *KIR2DL3 HLA-C1* homozygous patients. However, the question why these cells are more active remained unanswered.

Several receptors have been reported to be differentially expressed in HCV infection (reviewed in (Cheent et al. 2011)), however, the most consistent finding links increased frequencies of NKG2A^{+} NK cells to chronic HCV infection (Jinushi et al. 2004; Nattermann et al. 2005; Nattermann et al. 2006; De Maria et al. 2007; Ahlenstiel et al. 2010). Here we confirmed this finding and, more importantly, report that NK cells of seronegative IDUs are characterized by low frequencies of NKG2A^{+} NK cells. The simultaneous analysis of the expression of NKG2A and KIR2DL3 on the same cell revealed a distinct NK cell phenotype in seronegative IDUs possibly linking an expansion of KIR2DL3[+]NKG2A^{-} NK cells to protection from HCV infection. In addition we observe the inverse phenotype characterized by an expansion of KIR2DL3[-]NKG2A^{+} NK cells in IDUs with chronic HCV infection. We also found an inverse correlation between the expression of NKG2A and KIR2DL3, NKG2A and NKG2C as well as a positive correlation between KIR2DL3 and NKG2C. As described above, seronegative IDUs displayed significantly increased levels of NKG2C^{+} NK cells when compared to NK cells of chronically HCV infected IDUs alone. Although seronegative IDUs did no longer display significantly increased levels

of NKG2C when IDUs with resolved infection and healthy controls were taken into account as well, we still observed a strong trend towards increased NKG2C levels on NK cells of seronegative IDUs and IDUs with resolved infection, emphasizing a potential role of the C-type lectin-like binding receptors, NKG2A and NKG2C, in protection from HCV infection.

This notion is further strengthened by the upregulation of HLA-E, the NKG2A/NKG2C ligand, in liver biopsies of HCV infected patients and a direct correlation between viral load and HLA-E transcript levels that we report here. Interestingly, we did not observe an upregulation of HLA-E transcript levels in liver biopsies of HBV infected patients. As HCV induces a strong IFN-α response in the host while HBV does not, we hypothesized that HLA-E might be upregulated in an IFN-α dependent manner. While cytosolic viral RNA binds to RIG-I, which is believed to be the main sensor for HCV infection, TLR3 senses endosomal double stranded RNA (reviewed in (Horner et al. 2013)). Upon engagement it induces the activation of IRF3, ultimately causing the induction of type I interferons. We stimulated primary human hepatocytes with the TLR3 agonist poly I:C *in vitro* and observed upregulation of *HLA-E* transcript levels. HLA-E is stabilized at the cell surface by binding of a very restricted subset of peptides derived from the leader sequence peptides of other HLA class I molecules, thus increased *HLA-E* transcript levels alone do not necessarily result in increased HLA-E protein expression. However, we were able to induce HLA-E expression on primary human hepatocytes by direct IFN-α stimulation, suggesting that HLA-E is upregulated in the HCV infected liver in an IFN-α dependent manner, possibly resulting from sensing of double stranded RNA by TLR3.

Upregulation of HLA-E in HCV infected livers has previously been reported (Nattermann et al. 2005). In fact, Nattermann et al. describe an HCV peptide (core$_{35-44}$) actively stabilizing HLA-E on host cells, confirming the importance of HLA-E in HCV infection. Cheent et al. recently showed that core$_{35-44}$ not only stabilized HLA-E, but in the presence of HLA Class I leader peptides inhibits NK cells via NKG2A. The data also demonstrate that CD94 in the absence of NKG2A has a distinct specificity for HLA-E-peptide complexes. The combination of peptides that bind the

CD94/NKG2A heterodimer with those binding only CD94 results in a synergistic inhibition of NKG2A$^+$ NK cells (Cheent et al. 2013).

Here we propose a new model explaining the protective effect described for KIR2DL3. We suggest that KIR2DL3 itself might not be directly responsible for protection from HCV infection but rather serves as a marker for NKG2A and NKG2C expression. We tested the functionality of NK cells of seronegative IDUs and IDUs with chronic HCV infection in response to different NK cell target cell lines, mediating NK cell activation via NKG2D, NCRs or CD16 and found increased CD107a expression by NK cells of seronegative IDUs in response to 221 cells. However these models do not take the specific intrahepatic environmental changes during HCV infection into account, as HLA-E is not included in these systems. We therefore tested NK cell functionality in response to HLA-E expressing target cells and compared it to functionality in the absence of HLA-E. Total NK cells of healthy individuals were inhibited in the presence of HLA-E. A more detailed analysis of the different NK cell subsets revealed that KIR2DL3$^-$NKG2A$^+$ NK cells were inhibited in the presence of HLA-E, while KIR2DL3$^+$NKG2A$^-$ NK cells were not inhibited by HLA-E. However, when NK cells of healthy individuals were sorted and the functionality of the NKG2A enriched and NKG2A depleted NK cell fraction in response to HLA-E expressing and non-HLA-E expressing target cells was analyzed, we did not observe significant HLA-E mediated inhibition in the different fractions. This might be due to insufficient purity of the NKG2A sorted fractions using the Stem Cells technologies Easysept system, lack of accessory cells or impairment of NKG2A functionality by the antibody used to sort NKG2A$^+$ NK cells.

When we stimulated PMBCs of chronically HCV infected IDUs and seronegative IDUs with normal target cells and HLA-E expressing target cells we observed NK cell inhibition by HLA-E in NK cells from chronically HCV infected IDUs but not seronegative IDUs, indicating that the functionality of NK cells of seronegative IDUs is not impaired in the presence of HLA-E.

Collectively, our data strongly suggest that HCV seronegative IDUs are immunologically distinct by their NK cell population and to some extent protected from developing chronic hepatitis C infection. The protective effect is unlikely to prevent HCV entry and early viral replication. In fact, our proposed mechanism requires upregulation of HLA-E in the liver, which is observed upon HCV infection and is probably stimulated by type I or type III interferon. In the chimpanzee model high levels of interferon stimulated gene expression were observed within 2 days after HCV infection (Bigger et al. 2001; Park et al. 2012) whereas adaptive immune responses were delayed and became detectable only after 6-8 weeks (Thimme et al. 2002; Shin et al. 2011). In the setting of intrahepatic HLA-E upregulation, KIR2DL3$^-$NKG2A$^+$ NK cells, predominantly found in chronically HCV infected individuals, are potently inhibited allowing ongoing HCV replication and infection (Figure 5.1), while KIR2DL3$^+$NKG2A$^-$NK cells, characteristically found in seronegative IDUs, can readily respond before seroconversion as they are not inhibited by HLA-E (Figure 5.2).
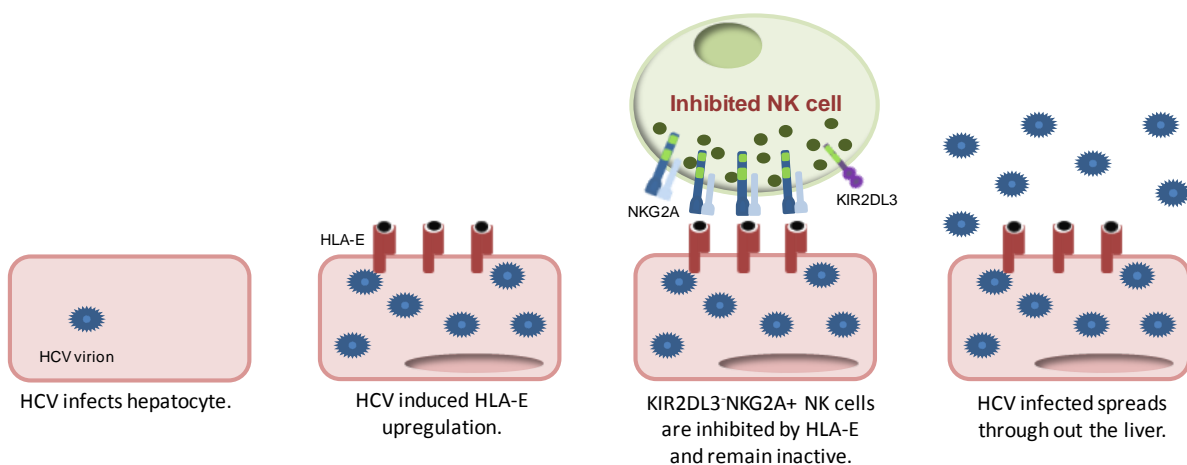
Figure 5.1: KIR2DL3$^-$NKG2A$^+$ NK cells are unable to protect from HCV infection.
HCV infection of hepatocytes leads to HLA-E upregulation. KIR2DL3$^-$NKG2A$^+$ NK cells are inhibited by HLA-E and do not kill infected hepatocytes resulting in viral replication and chronic HCV infection.

We hypothesize that less inhibition of NK cells from HCV seronegative IDUs during the very early phase of infection allows the eradication of the first wave of HCV infected hepatocytes even before humoral immune responses are induced. Evidence for viral replication is supported by detection of CD8 T cell responses against non-structural proteins in a subset of HCV seronegative IDUs (21.1%; data not shown).

Such T cell responses were previously reported in similar HCV exposed cohorts, however, in previous studies and also in our cohort the magnitude of these T cell responses was rather low compared to IDUs with spontaneously resolved HCV infection and are therefore unlikely to mediate the protective effect against HCV (Al-Sherbiny et al. 2005; Thurairajah et al. 2008; Thurairajah et al. 2011; Cameron et al. 2013).

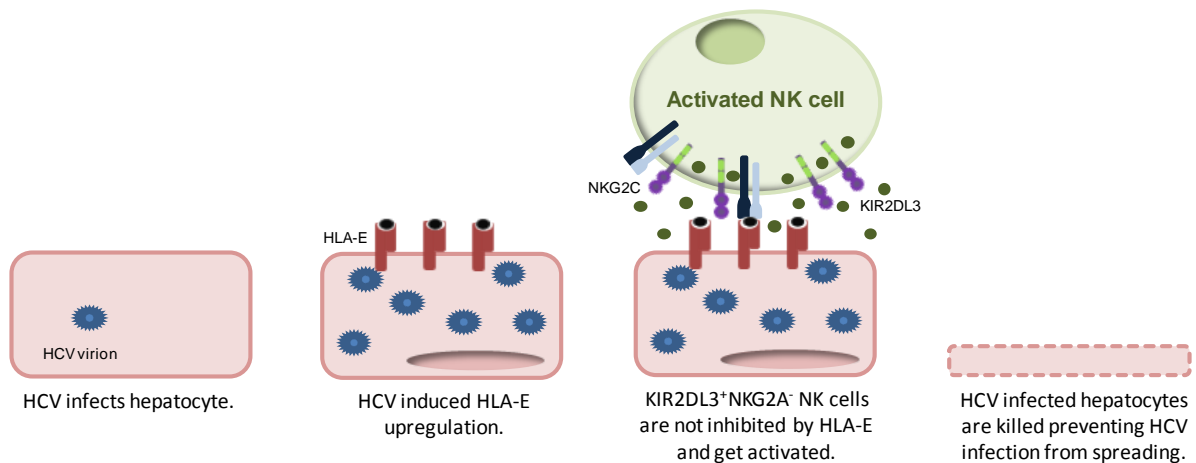**Figure 5.2: KIR2DL3+NKG2A- NK cells kill HCV infected hepatocytes.**
HCV induced HLA-E upregulation on hepatocytes does not inhibit KIR2DL3+NKG3A- NK cells. In addition they might be activated by interaction NKG2C and HLA-E. Infected hepatocytes are eliminated and HCV controlled before seroconversion.

Superior functionality of NK cells of seronegative IDUs in HCV infection might further be mediated by increased NKG2C and NKp46 levels on NK cells of seronegative IDUs compared to IDUs with chronic HCV infection. Although seronegative IDUs did no longer display significantly increased levels of NKG2C when all groups were included, the trend towards increased NKG2C levels on NK cells of seronegative IDUs and IDUs with resolved infection was still striking. As NKG2C also binds to HLA-E, increased degranulation or IFN-γ production might be expected by NK cells of seronegative IDUs in the presence of HLA-E. However, we did not observe increased functionality in this setting but merely absence of inhibition. This might be explained by the fact, that the affinity of HLA-E interaction is influenced by the bound peptide and is higher for NKG2A than NKG2C (Aldrich et al. 1994; Borrego et al. 1998). Although NKG2C on NK cells of seronegtive IDUs does not activate NK cells in the presence of HLA-E, it might still help to overcome the inhibiting signals mediated by NKG2A molecules on the same cell.

NK cells mature from CD56^{bright} to CD56^{dim} NK cells. This maturation process is associated with dynamic changes in the expression patterns of NK cell receptors. NKG2A is expressed on approximately 90% of CD56^{bright} NK cells and is lost when NK cells differentiate to CD56^{dim} NK cells. This loss of NKG2A is accompanied by acquisition of KIRs, NKG2C and CD57 as a marker for previously activated NK cells (Bjorkstrom et al. 2010; Lopez-Verges et al. 2010). Although the phenotype of the NK cells of seronegative IDUs resembles the phenotype of a mature NK cell, we did not find clear evidence that the elevated frequencies of KIR2DL3^{+}NKG2A^{-} NK cells in HCV seronegative IDUs are the consequence of an advanced differentiation state, as there was no statistically significant difference in subset distribution and CD57 expression. It seems plausible that the NK cell phenotype in this subgroup of IDUs is predominantly the result of differential NK cell licensing/education and is determined by genetic predisposition. For successful arming NK cells need to be licensed by ligation of KIRs with the relevant ligand. Studies in humans indicate that the genetically determined KIR/KIR-ligand combination impacts NK cell education and the functional responsiveness (Yawata et al. 2008; Schonberg et al. 2011). Expression of NKG2A was found to buffer the overall functional responses in KIR repertoires (Andersson et al. 2009). This is in line with the observation that the phenotype protective against HCV is associated with a distinct KIR/KIR-ligand genotype (Khakoo et al. 2004; Knapp et al. 2010; Knapp et al. 2011). Of note, there was a trend towards increased frequencies of CD57 expressing NK cells in the CD56^{dim} population of seronegative IDUs and IDUs with resolved HCV infection, indicating that an advanced differentiation state of NK cells may at least partly contribute to the phenotype. To address this conclusively larger cohorts need to be studied.

We included HCV seronegative IDUs from the ward for inpatient detoxification treatment of drug addicts as well as from the clinic for opiate substitution treatment. Given an HCV seroprevalence in IDUs from Germany of more than 80% and based on the reported risk behavior of this patient population (Scherbaum et al. 2009), exposures to HCV are likely to have occurred also in the HCV seronegative group. Due to the cross-sectional design of our study we were unable to analyze samples

from HCV-RNA positive IDUs prior to seroconversion. Importantly, our data suggest that the difference in NK cell phenotype is not simply the consequence of HCV infection or injection drug use as healthy controls showed an NK cell phenotype similar to IDUs with chronic infection. In previous studies different factors have been associated with HCV infection status in IDUs including age, duration of injection drug use and time in prison. This strongly suggests that the likelihood and frequency of exposures to HCV is an important factor that predicts the infection status. We also noted shorter duration of injection drug use in HCV seronegative IDUs (table 2.1). Although we cannot formally prove that HCV seronegative IDUs were indeed exposed to HCV, our data and previous studies support that the subgroup of seronegative IDUs is also biologically distinct and not solely the product of a different risk taking behavior (Golden-Mason et al. 2010; Knapp et al. 2010; Knapp et al. 2011; Warshow et al. 2012). The protective effect of the KIR2DL3$^+$NKG2A$^-$ NK cell population in HCV seronegative IDUs may not be absolute and the behavioral risk profile clearly contributes to the HCV infection status. It seems possible that the NK cell phenotype described here is beneficial upon exposures to low doses of HCV as it might be more common during needle sharing. This would be in line with previous genetic association status reporting a protective effect of homozygosity for the *KIR2DL3* gene allele and its ligand *HLA-C1* only in patients exposed to HCV by IDU but not in patients who received blood transfusion (Khakoo et al. 2004). In case of exposure to higher concentrations of HCV the protective effect may be overcome followed by acute hepatitis C.

5.2 NK cell memory, HCVM infection status and HCV infection outcome

Previously, mature NK cells in the periphery were considered a terminally differentiated effector cell population incapable of self-renewal. If a mature NK cell became activated and performed its effector duties, it was also thought to die within a half-life of approximately 2 weeks (Jamieson et al. 2004). This dogma has recently been challenged by several studies demonstrating that a long lived subset of previously activated NK cells exits (Pyzik et al. 2009; Paust et al. 2010; Sun et al. 2011; Abdul-Careem et al. 2012). Most prominently HCMV, a complex beta herpes virus capable of replicating in various cell types, has been reported to induce a long

lasting reconfiguration of the NK cell subset, characterized by expansion of NK cells expressing high levels of NKG2C (Guma et al. 2004). These NKG2C$^+$ NK cells are also characterized by low expression levels of NKG2A, NKp30 and NKp46 as well as increased expression of LILRP1 and KIRs (Muntasell et al. 2013). In addition Beziat et al. reported that HCMV seropositivity is associated with an expansion of CD56^{dim}NKG2C$^+$ NK cells in patients with chronic HBV or HCV infection. Multi-color flow cytometry revealed that the expanded CD56^{dim}NKG2C$^+$ NK cells displayed a highly differentiated phenotype, expressed high amounts of granzyme B and exhibited polyfunctional responses (CD107a, IFN-γ, and TNF-α) to stimulation with antibody-coated as well as HLA-E expressing target cells but not when stimulated with IL-12/IL-18. Importantly, CD56^{dim}NKG2C$^+$ NK cells had a clonal expression pattern of inhibitory KIR receptors specific for self-HLA class I molecules, with predominant usage of KIR2DL2/3 (Beziat et al. 2012).

Taken together, the NK cell phenotype induced in HCVM infection closely resembles the NK cell phenotype we describe as protective in HCV infection, suggesting a possible association between HCV infection outcome and HCMV infection status. To address this, we determined the CMV serostatus of 391 IDUs from our cohort. Our analysis revealed that 52% of IDUs chronically infected with HCV were seropositive for HCMV while 60% of IDUs with resolved and 59% of HCV seronegative IDUs were tested positive for anti-CMV. Although this difference is clearly not statistically significant, there is the expected tendency linking HCMV infection to HCV clearance and resistance. The relevance of this trend of higher CMV seroprevalence in IDUs protected from HCV infection is further strengthened when the age of IDUs is taken into account. The HCMV prevalence typically increases with age (Lubeck et al. 2010), however, our cohort of seronegative IDUs are in average significantly younger than chronically HCV infected IDUs. Moreover, we observed differences in the median time of drug consumption between the groups with different HCV infection outcome. Patients chronically infected with HCV report a median time of drug abuse of 15.3 years while IDUs with resolved HCV infection and seronegative IDUs report median times of drug abuse of 8.6 years and 6.7 years. To our knowledge there is no specific data on the HCMV seroprevalence in IDUs in Germany or a study linking

HCMV prevalence to duration of drug abuse. However, we would expect HCMV prevalence to increase with time of drug abuse. The patients included in this analysis were chosen based solely on their HCV infection status, however multiple factors are known to influence HCV infection outcome, including *IFNL3* genotype (Tillmann et al. 2010), *KIR2DL3* genotype (Khakoo et al. 2004; Knapp et al. 2010) and gender (Page et al. 2009). Further stratification of the included subjects with respect to known protective factors such as *IFNL3* or *KIR2DL3* genotype might give better insights into the interplay between HCV and HCMV infection. In addition a renewed expansion of NKG2C$^+$ NK cells has been reported to be likely in reactivation of HCMV infection (Guma et al. 2006) suggesting a possible link between the time point of HCMV infection and HCV clearance.

In summary, KIR2DL3$^+$NKG2A$^-$ NK cells are conclusively linked to favorable HCV infection outcome. Our data indicates that there might be more than one path to the generation of this favorable NK cell phenotype. While some patients have the KIR2DL3$^+$NKG2A$^-$ NK cell phenotype by genetic predisposition mediated by KIR2DL3 and HLA-C1 group alleles others may develop it as a result of encountering infections such as HCMV throughout their live. A prospective study analyzing potential changes of the NK cell phenotypes, HCV infection status and HCMV infection status over a longer period of time is needed and would allow to gain new insights into the dynamics of the interaction between NK cells and HCV infection.

6 Summary

Intravenous drug users (IDUs) are a major risk group for hepatitis C infection in Germany. In fact 73% of IDUs living in Germany are HCV seropositive while only 27% of IDUs are HCV seronegative. The frequently reported shared use of injection equipment suggests that the majority of HCV seronegative IDUs have also been exposed to HCV, raising the question why these IDUs do not seroconvert and if they might have some kind of natural resistance to HCV infection. NK cells represent a major subset of intrahepatic lymphocytes and certain NK cell phenotypes have been associated with HCV infection outcome. NK cells are regulated by a complex network of activating and inhibiting receptors. Interestingly, homozygosity for the inhibiting NK cell receptor gene *KIR2DL3* and its ligand *HLA-C1* has been associated with HCV clearance and resistance to HCV. However, the functional mechanisms underlying this protective effect by genetic predisposition remain elusive.

In this study we comparatively analyzed the phenotype and function of NK cells of IDUs with chronic HCV infection, resolved HCV infection and seronegative IDUs. Our results show that NK cells of seronegative IDUs are characterized by higher frequencies of NKp46, KIR2DL3 and NKG2C and lower frequencies of NKG2A. In line with a functional benefit of higher NKp46 expression levels, we observed increased CD107a production by NK cells of seronegative IDUs when NK cells were activated via NKp44 and NKp46. A more detailed analysis of NKG2A and KIR2DL3 revealed significantly higher frequencies of KIR2DL3^{+}NKG2A^{-} NK cells in HCV seronegative IDUs. In addition, we showed an inverse correlation between KIR2DL3 and NKG2A expression, suggesting that KIR2DL3 may potentially serve as a tag for NKG2A expression rather than playing a causal role for the previously described protective effects itself. Analysis of liver biopsy samples revealed increased transcription levels of HLA-E, the NKG2A ligand, in liver biopsies of HCV infected patients correlating with HCV viral load. *In vitro* experiments with primary human hepatocytes demonstrated that HLA-E transcription and protein expression were upregulated by IFN-α. To elucidate the influence of HLA-E on the KIR2DL3^{+}NKG2A^{-} NK cells of seronegative IDUs, we stimulated NK cells of healthy individuals with

HLA-E high and low expressing target cells and analyzed CD107a and IFN-γ production. Total NK cells and KIR2DL3⁻NKG2A⁺ NK cells were inhibited in the presence of HLA-E high expressing target cells, while KIR2DL3⁺NKG2A⁻ NK cells were not inhibited in the presence of HLA-E. Furthermore, we were able to show that NK cells of chronically HCV infected IDUs were potently inhibited in the presence of HLA-E while NK cells of seronegative IDUs showed no inhibition. We suggest a model in which HCV productively infects hepatocytes and causes type I interferon-mediated HLA-E upregulation. In this setting KIR2DL3⁻NKG2A⁺ NK cells, predominantly found in chronically HCV infected individuals, are potently inhibited by HLA-E allowing ongoing HCV replication and persistent infection, while KIR2DL3⁺NKG2A⁻ NK cells, characteristically found in seronegative patients, can readily respond and eliminate HCV infection before seroconversion.

Expansion of this "protective" NK cell phenotype in HCV seronegative IDUs could be the consequence of distinct NK cell education/licensing or a different NK cell differentiation stage. In favor of distinct education of NK cells in HCV seronegative IDUs we did not observe a statistically significant difference in CD57 expression as a marker of advanced differentiation of NK cells. This would be in line with previous genetic association studies linking a particular KIR/KIR-ligand genotype with favorable HCV outcome. However, there was at least a trend towards higher CD57 expression on NK cells of HCV seronegative IDUs, suggesting that an advanced differentiation state my partly contribute to the phenotype. Interestingly, the NK cell phenotype in HCV seronegative IDUs resembles the NK cell phenotype described upon HCMV infection. Collectively, our data indicate that there might be more than one path to the generation of the favorable KIR2DL3⁺NKG2A⁻ NK cell phenotype. While some patients have the NK cell phenotype by genetic predisposition others may develop it as a consequence of infections such as HCMV. A prospective study analyzing potential changes of the NK cell phenotypes, HCV infection status and HCMV infection status over a longer period of time is needed and would allow to gain new insights into the dynamics of the interaction between NK cells and HCV infection.

7 Zusammenfassung

Patienten mit intravenösem Drogenabusus („intravenous drug users", IDUs) stellen eine Hauptrisikogruppe für Hepatitis-C-Virusinfektionen in Deutschland dar. In Deutschland sind 73% aller IDUs HCV seropositiv und nur 27% HCV seronegativ. Da Injektionsutensilien häufig von mehreren Personen geteilt werden, sind mit hoher Wahrscheinlichkeit nahezu alle IDUs einschließlich der seronegativen Patienten HCV exponiert. Dies wirft die Frage auf, warum diese Gruppe der IDUs keine akute Infektion entwickelt und ob sie eine Art „natürliche Resistenz" gegen eine HCV-Infektion haben.

NK-Zellen sind eine wichtige Population intrahepatischer Lymphozyten und unterschiedliche NK-Zellphänotypen wurden bei unterschiedlichen HCV-Infektionsverläufen beschrieben. NK-Zellen werden durch ein komplexes Netzwerk aus aktivierenden und inhibierenden Rezeptoren reguliert. Patienten, die für das Gen des inhibierenden NK-Zellrezeptors *KIR2DL3* und seinen Liganden *HLA-C1* homozygot sind, haben eine erhöhte Wahrscheinlichkeit, eine HCV-Infektion spontan auszuheilen oder erst gar nicht infiziert zu werden. Allerdings bleibt der funktionelle Mechanismus für diesen protektiven Effekt ungeklärt.

In dieser Studie haben wir den Phänotyp und die Funktion der NK-Zellen von IDUs mit chronischer HCV-Infektion, ausgeheilter HCV-Infektion und HCV seronegativen IDUs vergleichend analysiert. Unsere Ergebnisse zeigen, dass die NK-Zellen HCV seronegativer IDUs durch höhere Frequenzen von NKp46, KIR2DL3 und NKG2C sowie einer niedrigeren Frequenz von NKG2A charakterisiert sind. In Einklang mit der erhöhten NKp46 Frequenz wiesen NK-Zellen von HCV seronegtiven IDUs einen funktionellen Vorteil durch erhöhte CD107a Produktion auf, wenn sie über NKp44 und NKp46 stimuliert wurden. Eine detaillierte Analyse von NKG2A und KIR2DL3 zeigte statistisch signifikant erhöhte Frequenzen von KIR2DL3$^+$NKG2A$^-$ NK Zellen bei HCV seronegativen IDUs. Zusätzlich konnten wir eine inverse Korrelation zwischen der Expression von KIR2DL3 und NKG2A zeigen. Es erscheint daher möglich, dass KIR2DL3 lediglich als Marker für eine niedrige NKG2A-Expression

fungiert und selbst gar nicht für den ihm zugeschrieben protektiven Effekt verantwortlich ist. Die Analyse von Leberbiopsien HCV-infizierter Patienten zeigte eine erhöhte Transkription des NKG2A-Liganden HLA-E sowie eine Korrelation mit der Höhe der Viruslast. In primären humanen Hepatozyten wurde sowohl die HLA-E Transkription als auch die Proteinexpression durch Stimulation mit IFN-α erhöht. Um den Einfluss von HLA-E auf die KIR2DL3$^+$NKG2A$^-$ NK-Zellen seronegativer IDUs weiter zu untersuchen, wurden NK-Zellen gesunder Individuen mit aktivierenden Zellen stimuliert, die entweder eine hohe oder eine niedrige HLA-E-Expression aufwiesen. In der Analyse von CD107a und IFN-γ Expression zeigte sich, dass die Gesamtpopulation der NK-Zellen sowie die Teilpopulation der KIR2DL3$^-$NKG2A$^+$ NK-Zellen in Anwesenheit von HLA-E inhibiert werden. Im Gegensatz dazu wurden die KIR2DL3$^+$NKG2A$^-$ NK-Zellen nicht durch HLA-E inhibiert. Weiterhin konnten wir zeigen, dass NK-Zellen von chronisch mit dem HCV infizierten IDUs aber nicht NK-Zellen von HCV seronegativen IDUs wirksam durch die Anwesenheit von HLA-E inhibiert wurden. Wir schlagen hier ein Modell vor, in dem das HCV Hepatozyten infiziert und über Induktion von Typ I Interferonen HLA-E auf Hepatozyten hochreguliert. In diesem System werden KIR2DL3$^-$NKG2A$^+$ NK-Zellen, die vorwiegend in chronisch infizierten Patienten gefunden werden, effizient durch HLA-E gehemmt, was eine weitere HCV Replikation ermöglicht und in einer persistierenden Infektion resultiert. Im Gegensatz dazu können KIR2DL3$^+$NKG2A$^-$ NK-Zellen der HCV seronegativen IDUs aktiviert werden und die Infektion kontrollieren, bevor es zur Serokonversion kommt.

Die Expansion dieser „protektiven" NK-Zellen könnte die Folge einer speziellen NK-Zell-Lizensierung oder einer veränderten Differenzierung sein. Für die spezielle Lizensierung spricht, dass wir keine statistisch signifikanten Unterschiede bei dem Differenzierungsmarker CD57 feststellen konnten. Die spezielle Lizensierung wäre auch im Einklang mit der genetischen Assoziation zwischen bestimmten KIR/KIR-Liganden-Kombinationen und einem vorteilhaften HCV-Infektionsverlauf. Allerdings war zumindest eine Tendenz zu erhöhter Expression von CD57 auf NK-Zellen von seronegativen IDUs erkennbar, so dass ein fortgeschrittener Differenzierungsgrad der NK-Zellen zum „protektiven" Phänotyp beitragen könnte. Interessanterweise

72

ähnelt der NK-Zellphänotyp der HCV seronegativen IDUs dem Phänotyp nach einer HCMV Infektion. Zusammengefasst, weisen unseren Daten darauf hin, dass es mehr als einen Weg zu dem günstigen $KIR2DL3^+NKGA^-$ NK Zell Phänotyp geben könnte. Während einige Patienten diesen Phänotyp als Konsequenz ihrer Immungenetik haben, resultiert er bei anderen möglicherweise aus einer Infektion zum Beispiel mit CMV. Eine Studie, die die Dynamik des NK-Zell-Phänotyps, sowie den HCV- und HCMV-Infektionsstatus über einen längeren Zeitraum untersucht, würde neue Einsichten in die Rolle von NK-Zellen bei der HCV-Infektion ermöglichen.

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9 Abbreviations

221	LCL721.221 cells
TLR3	toll-like receptor 3
°C	degree Celsius
µ	micro
aa	amino acids
ADCC	antibody dependent cellular cytotoxicity
APC	allophycocyanin
APC Cy7	APC-Cyanine7
CD	cluster of differentiation
cDNA	complementary DNA
CH	chronic
CLDN1	claudin-1
cLDs	cytoplasmic lipid droplets
CMV	cytomegalovirus
DC	dendritic cells
DGAT-1	diacylglycerol acyltransferase-1
DNA	deoxyribonucleic acid
ds	double stranded
E	envelope
EGFR	epidermal growth factor receptor
eIF2α	α subunit of eukaryotic initiation factor 2
ER	endoplasmatic reticulum
et al.	and others (*lat.* Et alii)
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
h	hour
H	healthy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus

HLA	human leukocyte antigen
IDU	intravenous drug users
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IRES	internal ribosome entry site
IRF-3	interferon regulatory factor-3
ISGs	IFN-stimulated genes
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
K562	human acute myelocytic leukemia cell line K562
kb	kilo bases
KIR	killer cell immunoglobulin-like receptor
LCMV	lymphocytic choriomeningitis virus
LDL	low-density lipoproteins
LVPs	lipo-viro-particles
m	milli
M	molar
MAVS	mitochondrial antiviral signaling proteins
MHC	major histocompatibility complex
min	minute
mRNA	messenger RNA
NCR	natural cytotoxicity receptors
NF-κB	nuclear factor-κB
NK cell	natural killer cell
nm	nanometer
NS	non structural
OCLN	Occludin
ORF	open reading frame
p815	murine lymphoblast-like mastocytoma cell line p815
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell

PCR	polymerase chain reaction
PD-1	programmed death 1
PE	phycoerythrin
PHH	primary human hepatocyte
PKR	protein kinase R
poly I:C	polyinosine-cytosine
PRR	pattern recognition receptor
R	resolver
RIG-I	retinoic acid inducible gene-I
RNA	ribonucleic acid
SN	seronegative
SNPs	single nucleotide polymorphisms
SR-BI	scavenger receptor class B type I
SVR	sustained viral response
TNF	tumor necrosis factor
TRIF	TIR-domain-containing adaptor-inducing interferon-β
ULBP	UL16-binding protein
UTR	untranslated regions
VLDL	very-low-density lipoproteins
WHO	world health organization

10 List of figures

11 List of tables

12 Publications

Manuscript in revision in "*Journal of Hepatology*":

A distinct NK Cell phenotype associated with less HLA-E-mediated inhibition protects against productive Hepatitis C Virus infection

Christine Thoens, Christoph Berger, Martin Trippler, Holger Siemann, Melanie Lutterbeck, Ruth Broering, Jörg Schlaak, Falko. M. Heinemann, Andreas Heinold, Jacob Nattermann, Norbert Scherbaum, Galit Alter, Joerg Timm

12.1 Presentations

23rd annual meeting of the German society for Virology

"HCV seronegative injection drug users are characterized by higher frequencies of KIR2DL3$^+$ NKG2A$^-$ NK cells"

Kiel, Germany; Oral presentation

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14 Curriculum Vitae

Der Lebenslauf ist aus Gründen des Datenschutzes

in der Online-Version nich enthalten.

105

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15 Declaration (Erklärung)

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Analysis of NK cells in intravenous drug users exposed to Hepatitis C" zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Christine Thöns befürworte.

Essen, den _____   _____   _____

Prof. Dr. med. Jörg Timm

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

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Christine Thöns