The role of Natural Killer cells and Natural Killer T cells in HCV infection

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie

an der

Universität Duisburg-Essen

vorgelegt von

Tina Senff

aus Schmalkalden

August 2017

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden am Institut für Virologie der Universität Duisburg-Essen und am Institut für Virologie der Heinrich-Heine-Universität Düsseldorf durchgeführt.

1. Gutachter: Prof. Dr. Jörg Timm
2. Gutachter: Prof´in. Dr. Astrid Westendorf
3. Gutachter: Prof. Dr. Jacob Nattermann

Vorsitzender des Prüfungsausschusses: Prof. Dr. Daniel Hoffmann

Tag der mündlichen Prüfung: 12.01.2018

“The universe is big. It’s vast and complicated and ridiculous.
And sometimes, very rarely, impossible things just happen
and we call them miracles”
Dr. Who

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1 Introduction

1.1 Hepatitis C virus (HCV) infection

Initially, Hepatitis C was described as a transfusion-associated hepatitis which was neither hepatitis A nor hepatitis B, thus was termed non-A non-B hepatitis (NANBH) (Feinstone et al. 1975). Almost 15 years later in 1989 the pathogen causing NANBH was isolated by Choo et al. (1989) and the identified positive stranded ribonucleic acid (RNA) virus was named hepatitis C virus (HCV). HCV can cause acute as well as chronic infection. After an incubation period of two weeks up to six months only approximately 20% of infected individuals exhibit usually mild symptoms like fever and nausea, while the vast majority of acute HCV infections remains asymptomatic. Chronic disease can cause severe liver disease including liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Therefore, HCV is the major reason for liver transplantation in developed countries. To date, approximately 71 million people worldwide are chronically infected with HCV accounting for 1% of the population and the World Health Organization (WHO) estimates 1.75 million new HCV infections per year. HCV is still a major public health burden and each year approximately 400 000 people die from liver cirrhosis and HCC caused by HCV infection. The worldwide distribution of HCV is uneven with the highest prevalence of 2.3% in the Eastern Mediterranean Region and the European Region (1.5%) while the lowest prevalence can be observed in the South-East Asian Region with 0.5% (WHO 2017).

1.1.1 HCV structure

HCV is a positive stranded enveloped RNA virus of the Flaviviridae family, which also comprises dengue, yellow fever and west Nile virus. Besides HCV only the GB-virus B (Adams et al. 2017), the nonprimate (NPHV) (Kapoor et al. 2011), rodent (RHV) (Kapoor et al. 2013) bat (Quan et al. 2013) and the bovine hepacivirus (Baechlein et al. 2015) are grouped into the Hepacivirus genus. HCV has a length of approximately 9.6 kilo bases (kb) and the genome is encoding for a single long open reading frame. The encoded polyprotein is proteolytically cleaved into three structural (core, E1, E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). Short untranslated regions (5´-UTR and 3´-UTR) which contain secondary RNA structures, needed for translation and replication of the genome, are flanking the approximately 3 000 amino acid long open reading frame. The 5´-UTR

contains an internal ribosome entry site responsible for the initiation of protein translation. Two functional modules are encoded by the HCV genome. While the assembly module comprises the two envelope proteins E1 and E2, the core protein, p7 as well as NS2, the other non-structural proteins are required for RNA replication and form the replication module (reviewed in Penin et al. 2004, and Bartenschlager et al. 2013)

1.1.2 HCV genotypes

HCV shows substantial genetic variability due to its high replication rate paired with a high error rate of the RNA-dependent RNA polymerase. The high error rate caused by the lack of proof reading function of the HCV RNA polymerase results in 10^{-3} base substitutions per site per year (Major et al. 1999). Together with the high turnover rate of approximately 10^{12} virions per day this leads to the generation of a highly heterogenous pool of viruses within an infected individual (Neumann et al. 1998). The HCV quasispecies represents the entirety of all viral variants in a single individual and the viral variants are phylogenetically closely related but show a sequence variability (Martell et al. 1992). Moreover, based on the genetic distances in phylogenetic trees HCV can be classified into different genotypes. Until now seven major HCV genotypes numbered from one to seven have been reported and multiple subtypes named alphabetically have been identified. HCV strains between genotypes differ at 30-35% (Okamoto et al. 1992, Simmonds et al. 2005) whereas subtypes vary at <15% of nucleotide sites (Smith et al. 2014). Worldwide genotypes 1 and 3 are the most common ones and calculations estimated that they account for respectively 46% and 30% of HCV cases (Messina et al. 2015). The seven described genotypes show distinct geographical distribution with genotype 1 having the highest prevalence worldwide and being the most common genotype in Europe (50-70%) and North and South America (70%). Genotype 2 is the most common genotype in West Africa while genotype 4 is most prominent in Central and North Africa. In South and Southeast Asia genotype 3 and 6 are predominant. Accounting for less than 1% of the HCV cases genotype 5 mostly described in Southern and Eastern Sub-Saharan Africa, is responsible for the fewest infections besides genotype 7, which until now has only been reported in a few patients originating from the Democratic Republic of Congo (reviewed in Scheel et al. 2013, Messina et al. 2015, Murphy et al. 2015). These genotypes are not only associated with distinct geographical distribution but

also with different sensitivity to treatment, for instance, to interferon alpha (IFNα) therapy which was standard of care for a long time.

1.1.3 HCV therapy

Since some people are able to clear HCV spontaneously, HCV therapy is not always required during acute infection. In chronically HCV infected patients, the main goal of therapy is to cure the infection and therefore prevent HCV-related causes like HCC and liver cirrhosis. Successful treatment results in a sustained virological response (SVR) characterised by the absence of detectable HCV RNA after a defined period of time. The rate of SVR is strongly dependent on the treatment regimen and duration, the viral genotype and the stage of liver fibrosis. During the last few years, the launch of direct-acting antivirals (DAAs) changed HCV therapy dramatically. Before that, recombinant IFNα was the main component in HCV therapy regimens. Since then, therapy was stepwise improved by the combination of IFNα with ribavirin, the modification of INFα or the combination with protease inhibitors (reviewed in Pawlotsky et al. 2015). IFNα was administered as a HCV therapy for the first time in 1986, when HCV was still known as NANBH (Hoofnagle et al. 1986). In addition to the severe side effects of IFNα therapy, SVR rates were extremely low (approximately 10%). However, combination treatment of IFNα with the nucleoside analogue ribavirin could increase overall SVR rates up to approximately 30%. Furthermore, modification of IFNα by the addition of polyethylene glycol (PEG) significantly increased the elimination half-life of IFNα and when administered in combination with ribavirin SVR rates over 40% were observed. Almost 10 years after the launch of PEGylated IFNα, PEGylated IFNα and ribavirin were combined with the first generation of DAAs. Treatment with the first-wave of protease inhibitors blocking the viral replication by targeting the active site of the NS3/4A protease resulted in SVR in approximately 70% of infected individuals (reviewed in Pawlotsky et al. 2015). Between 2011 and 2015 various DAAs were licensed and administered in different HCV therapy regimens, further improving SVR rates. Sofosbuvir, a nucleotide analogue inhibiting the NS5B polymerase, was the first DAA reaching a SVR rate of over 90% (Lawitz et al. 2013). In addition, the first pan genotypic drug Epclusa, a combination of sofosbuvir and the NS5A inhibitor valpatasvir, was launched and approved for genotypes one to six in 2016. SVR rates over 95% were achieved, thus nearly all HCV infected individuals are curable (Feld et al. 2015). Even though pan-genotypic therapies are nowadays licensed, most treatment options are still

genotype-specific, therefore, HCV genotyping before treatment onset is required. Although a variety of DAAs is available, a few patients still do not achieve SVR which has been linked to the occurrence of resistance associated substitutions (RAS). RAS affect for instance the SVR rate to treatment with NS5A inhibitors especially in treatment experienced and cirrhotic patients (Zeuzem et al. 2017). Therefore, HCV resistance testing can support treatment decisions under certain conditions. Identification of resistance associated mutations at baseline results in the selection of presumably efficient therapy options (Kalaghatgi et al. 2016).

1.1.4 HCV transmission

HCV is a bloodborne virus and transmitted through infected blood or blood products. Before the late 1980s and therefore prior to the availability of serological and molecular tests for HCV, the main risk factor for acquiring HCV, was the transfusion of blood products. Since then, blood products are screened for HCV-RNA and HCV transmission through this route is therefore rarely reported. Nowadays, injection drug use is the most common cause of HCV transmission in western industrialised countries (reviewed in Alter 1997, WHO 2017).

1.1.5 HCV infection in people who inject drugs

Due to the common practice of people who inject drugs (PWID) to share needles or other injection materials the HCV transmission rate in this group of individuals is particularly high. Globally intravenous drug use is a major public health issue and reports estimate 15.9 million people worldwide being PWID (Mathers et al. 2008). Worldwide an estimate of 10 million PWID have detectable antibodies against HCV. Cumulatively, China, the USA and Russia account for 44% of HCV-RNA positive PWID worldwide. Especially in high income countries, the estimated frequency of HCV transmissions caused by intravenous drug use is tremendously high (80%) (Nelson et al. 2011). This is also evident in Germany, as the majority (76.2%) of all newly reported HCV infections by the Robert Koch-Institut (RKI) in 2016, where the transmission route was accounted for, were caused by intravenous drug use (figure 1.1) (Robert Koch-Institut 2016).

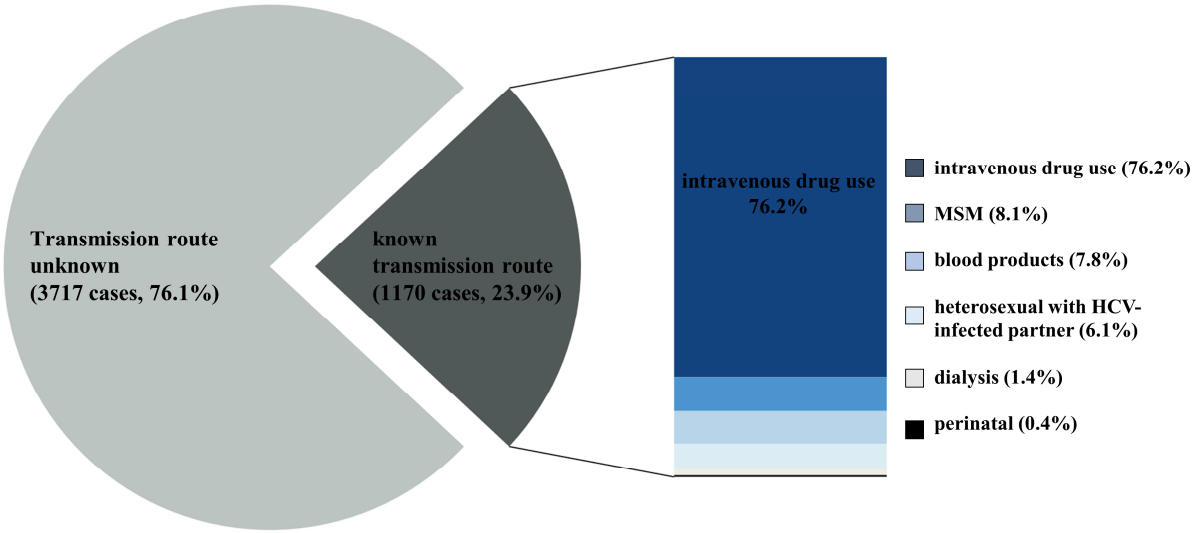

Figure 1.1: HCV transmission routes in Germany

In Germany 4887 initial HCV infections were diagnosed in 2015. In 76.1% of HCV cases the transmission route was unaccounted for, while in 23.9% the transmission route was reported. From the 1170 newly infected individuals 76.2% acquired HCV due to intravenous drug use. The second most frequent transmission route with 8.1% was men having sex with men (MSM). The remaining 15.7% of newly diagnosed HCV infections were either transmitted during dialysis, heterosexually transmitted from a HCV infected partner, acquired by transfusion of blood products before routine HCV testing was implemented or transferred perinatal (Robert Koch-Institut 2016).

Prevalence of HCV infection measured as antibody positivity among PWID in Europe ranges from 21% in Finland up to 90.5% in Estonia (Nelson et al. 2011). The recently reported prevalence rate of HCV infection among PWID in Germany is 63% including 44% of the total PWID having detectable HCV RNA. Depending on the geographic location reported HCV infection rates in Germany vary from 37% to 73% (Robert Koch-Institut Berlin 2016).

HCV is efficiently transmitted by intravenous drug use since the infection rate in PWID during the first two years after onset is approximately 29%. With increasing duration of intravenous drug use the prevalence of HCV positive individuals is steadily rising, reaching 72% after 10 years of drug abuse (Robert Koch-Institut Berlin 2016).

Neither spontaneous resolution of HCV infection nor treatment induced clearance protects from HCV reinfection, thus HCV infection does not confer immunity to subsequent infections. HCV reinfections are reported especially in high-risk populations like PWID, HCV-Human immunodeficiency virus (HIV) coinfected individuals and MSM (Lambers et al. 2011, Islam et al. 2017). In PWID reinfection

rates are also dependent on several factors such as age, gender and injection behaviour, like frequency of injections, sharing of injection materials and simultaneous multidrug use as well as imprisonment, or social factors such as residential stability, employment and social support (Grebely et al. 2012, Marco et al. 2013, Islam et al. 2017). A reduction of reinfection risk is seen in PWID engaging in opioid substitution therapy and mental health counselling (Islam et al. 2017). Although previous infections do not confer immunity to HCV infection, there are indications for a trend towards lower HCV reinfections in individuals who spontaneously cleared the infection in comparison to successfully treated individuals (Ingiliz et al. 2017). Furthermore, spontaneous resolution of the primary HCV infection leads to a partial protection as the duration and the maximum level of viremia are lower during a secondary HCV infection compared to the initial infection. The higher clearance rate of secondary infections is accompanied by an increased occurrence of cross-reactive neutralising antibodies against HCV and a broadened cellular T cell immune response (Osburn et al. 2010). The high-risk behaviour of PWID does not only lead to high reinfection rates but, due to the frequent exposure to HCV, mixed infections can occur where multiple viral strain are detectable at the same time in a single individual (Pham et al. 2010, Grebely et al. 2012).

An epidemiological study on PWID in Essen reported 45.2% being chronically HCV infected determined by presence of detectable HCV-RNA, whereas 27.9% resolve the infection consistent with absence of HCV-RNA and detectable HCV antibodies. The remaining 26.9% are anti-HCV negative as well as HCV-RNA negative (Robert Koch-Institut 2012). The described high-risk behaviour in PWID suggests that the anti-HCV negative group has likely been exposed to HCV but is able to clear infection prior to seroconversion.

1.1.6 Interferons and HCV infection

As IFNα administration was efficient in treatment of NANBH, the importance of interferons (IFNs) in HCV infection has been reported even before the term HCV was introduced (Hoofnagle et al. 1986). Three major classes of IFNs are described at the moment: type I, type II and type III IFNs. Type I IFNs comprise IFNα, IFNβ, IFNκ, IFNε and IFNω while type III IFNs include IFNλ1 to IFNλ4. Both groups are essential for the innate immune response and signal through the JAK-STAT pathway which results in the induction of interferon-stimulated genes (ISGs). These ISGs have antiviral and immunomodulatory roles. In HCV infection, viral RNA is sensed either in

the cytosol by retinoic acid inducible gene I (RIG-I) or the melanoma differentiation-associated protein 5 (MDA-5) or in the endosome through the Toll-like receptor 3 (TLR3). RIG-I mediated sensing, for instance, depends on the binding to a poly-(U/UC) region found in HCV in the 3´UTR. RNA sensing results in downstream signalling and the induction of type I as well as type III IFNs (Saito et al. 2008, Wang et al. 2009, Cao et al. 2015).

Treatment of hepatocytes with IFNα leads to an increase in ISG expression and an inhibition of HCV replication (Castet et al. 2002). This upregulation of ISGs is also induced by treatment with PEGylated IFNα in patients achieving SVR whereas non-responders already show high expression of ISGs at baseline. In non-responders additional administration of IFNα does not result in a further increase of ISG expression because the IFN signalling pathway shows a reduced responsiveness to IFNs (Sarasin-Filipowicz et al. 2008). Moreover, a single nucleotide polymorphism (SNP) (rs12979860) upstream of the *IFNλ3* locus is a strong predictor of achieving SVR to IFNα treatment. Furthermore, the C/C genotype of this SNP is an independent predictor for spontaneous clearance of HCV infection (Thomas et al. 2009, Tillmann et al. 2010, Fitzmaurice et al. 2015). The proinflammatory and antiviral cytokine IFNλ3 is upregulated in HCV infected liver and an increase in endogenous production of IFNλ by HCV infected hepatocytes has been reported (Marukian et al. 2011, Park et al. 2012). IFNλ3 is able to inhibit HCV virus replication by phosphorylating STAT1 and STAT2 leading to the activation of further signalling in the JAK-STAT pathway and transcription of ISGs in a similar manner like IFNα (Zhang et al. 2011). Furthermore, rs12979860 upstream of the IFNλ3 locus is in a high linkage disequilibrium with a dinucleotide variant that is the basis for IFNλ4. This dinucleotide variant, first described in HCV patients, (ss469415590) is a strong genetic predictor for HCV clearance as well (Prokunina-Olsson et al. 2013). While the CC variant of the IFNλ3 SNP is beneficial during acute HCV infection (Thomas et al. 2009), the SNP is associated with increased risk of liver fibrosis in chronic HCV infection (Eslam et al. 2015). Recently, it was identified that zinc inhibits the binding of IFNλ3 to IFNL receptor 1, providing evidence that zinc can mediate anti-inflammatory effects in HCV infection and might be effective against IFNλ3 induced liver damage in chronic infection (Read et al. 2017).

However, HCV has evolved mechanisms to evade the antiviral IFN system. The non-structural protein NS3/4A targets and cleaves the mitochondrial antiviral

signalling proteins that are important in the signalling of RIG-I and MDA5 and therefore prevents recognition and downstream signalling. Moreover, the NS3/4A protease blocks the TLR3 signalling by targeting TRIF which results in the inhibition of IFN production (reviewed in Morikawa et al. 2011, and Heim et al. 2014).

1.1.7 The adaptive immune system in HCV infection

Both the innate as well as the adaptive immune system is essential for eliminating HCV during acute infection. In contrast to the early induction of type I and III IFNs, virus specific T cell responses are detected in the liver weeks after infection (Thimme et al. 2002). Multiple studies show that virus specific $CD4^+$ as well as $CD8^+$ T cells are crucial for spontaneous resolution of HCV and that a lack of a strong T cell response results in virus persistence. In addition, HCV specific memory cells can persist after resolution and respond rapidly upon a second infection. A strong and sustained T cell response targeting a broad range of HCV epitopes is essential for HCV elimination during acute infection (Lechner et al. 2000, Thimme et al. 2001, Thimme et al. 2002). T cell depletion studies in chimpanzees demonstrated the importance of $CD8^+$ as well as $CD4^+$ T cells in the control of HCV infection (Grakoui et al. 2003, Shoukry et al. 2003). Thus, the help of $CD4^+$ T cells is needed to maintain effective $CD8^+$ T cell responses. As demonstrated in a chimpanzee model, animals that clear the infection have a strong cytotoxic T lymphocyte (CTL) response targeting several epitopes whereas animals that develop chronic infection display a weak CTL response during acute infection (Cooper et al. 1999). Viral clearance through HCV-specific $CD8^+$ T cells is mainly attributed to their high capability to secrete antiviral IFNγ upon activation (Frese et al. 2002, Jo et al. 2009). In addition, HCV-specific $CD8^+$ T cells efficiently secrete TNFα and are capable of directly killing virus infected cells via perforin or the Fas/Fas ligand (FasL) pathway (Ando et al. 1997). Immunogenetic studies revealed associations between certain human leukocyte antigen (HLA) class I haplotypes presenting viral antigens to T cells and spontaneous resolution, indicating the importance of virus specific $CD8^+$ T cell responses for infection outcome. HLA-B*27 and HLA-B*57 have been repeatedly associated with spontaneous immune control and a robust IFNγ response (McKiernan et al. 2004, Kim et al. 2011, Fitzmaurice et al. 2015).

Failure of controlling HCV replication during acute infection is associated with exhaustion of T cells after initial priming and expansion. Exhaustion is driven through continuous stimulation of $CD8^+$ T cells with viral antigens. $CD8^+$ T cell exhaustion is

characterised by impaired antiviral functionality as well as increased expression of numerous inhibitory receptors such as PD1, Tim-3, CTLA-4 and 2B4 (reviewed in Timm et al. 2015). In comparison to the periphery, intrahepatic T cells display an even higher expression of exhaustion markers (Kroy et al. 2014). Furthermore, exhausted $CD8^+$ T cells have a low proliferative potential (Kroy et al. 2014), an impaired ability to produce IFNγ and TNFα as well as a reduced cytotoxicity (Penna et al. 2007). Multiple studies addressed a possible reversion of the exhausted phenotype to restore HCV-specific $CD8^+$ T cell function. Anti-PDL1 antibodies blocking the PD1/PDL1 interaction enhance HCV-specific $CD8^+$ T cell function (Penna et al. 2007) however, to restore full antiviral functionality combined blockade of inhibitory receptors is required (Nakamoto et al. 2009).

Evasion of immune recognition is a common phenomenon in HCV infection since the majority of individuals develop chronic infection. One mechanism of HCV to evade T cell recognition is by selection of mutations inside as well as in the flanking regions of the targeted epitope. Three different molecular viral escape mechanisms are described that lead either to the complete abolishment or reduced $CD8^+$ T cell activation through the TCR peptide MHC class I complex. First, mutations within flanking regions of an HCV CD8 T cell epitope are able to alter the peptide processing in the proteasome by cleavage of the polypeptide inside the epitope sequence. However, correct proteasomal processing does not guarantee T cell response as mutations within the MHC class I binding sites can alter the binding affinity. Mutations within anchor residues result in unstable MHC class I/peptide complexes and regularly to an abolished presentation to the TCR through the loss of the epitope. Furthermore, mutations that occur inside the sequence that is recognised by the TCR can result in the reduction or the absence of a CD8 T cell response even though the epitope is properly cleaved and presented (reviewed in Holz et al. 2015). These viral escape mechanisms that perturb peptide binding to the MHC molecule and impair TCR recognition hinder the generation of a TCR-MHC class I complex which is able to activate $CD8^+$ T cells to elicit a IFNγ response that is sufficient to control HCV infection. To fully escape T cell recognition by HLA-B*27, multiple substitutions within the immunodominant epitope are required, possibly explaining the protective effect of this allele in HCV infection outcome (Dazert et al. 2009). Besides viral sequence mutations, a narrow TCR repertoire displaying a low diversity within the CDR3 region, is associated with immune escape in a chimpanzee

model, whereas the generation of a broadly cross-reactive TCR repertoire possibly prevents viral escape (Meyer-Olson et al. 2004). However, escape mutations can affect the viral fitness, thus reducing viral replication capacity (Neumann-Haefelin et al. 2008, Salloum et al. 2008, Dazert et al. 2009). As a result, the quasispecies generally comprises viral variants which balance viral replication and immune evasion (Uebelhoer et al. 2008). In the absence of immune pressure, reversion back to the prototype sequence occurs, which is advantageous for viral replication (Timm et al. 2004, Ray et al. 2005, Neumann-Haefelin et al. 2008).

In comparison to T cells as part of the adaptive immune system, the innate immune system is the first line of defence which is induced directly after infection and Natural Killer (NK) cells are the major innate cell type for mediating antiviral effects during HCV infection.

1.2 Natural Killer (NK) cells

NK cells have been defined in 1975 as large granular lymphocytes with the ability to kill target cells without prior priming and without restriction to the MHC expression on the target cell. Kiessling et al. (1975) showed that mouse NK cells are capable of killing Moloney leukaemia cells *in vitro*. NK cells comprise approximately 15% of the lymphocyte population in the periphery and are traditionally defined by their cell surface phenotype, lacking the T cell linage marker CD3 but expressing CD56. Nowadays NK cells are not solely defined by the expression of CD56 but furthermore by CD16. Based on the cell surface density of CD56 and the expression of CD16, NK cells are divided into three subtypes: $CD56^{dim,}$ $CD56^{bright}$ and $CD56^{negative}$. $CD56^{dim}$ NK cells account for the majority of NK cells (90%) defined by a low-density expression of CD56 and high surface expression of CD16. Whereas around 10% of NK cells in the periphery are $CD56^{bright}$, the third subset of $CD56^{negative}$ $CD16^{positive}$ NK cells is very rare (reviewed in Cooper et al. 2001, and Caligiuri 2008). These NK cell subsets have been associated with different functional properties and distinct differentiation stages in NK cell development (figure 1.2). $CD56^{dim}$ NK cells have a higher cytotoxic activity than $CD56^{bright}$ NK cells, thus produce more cytolytic granules. Through their high cell surface expression of CD16, $CD56^{dim}$ NK cells are efficient in CD16 mediated antibody dependent cell cytotoxicity (ADCC) in contrast to $CD56^{bright}$ NK cells. On the other hand, $CD56^{bright}$ NK cells are the subset most efficient in production of cytokines like IFNγ, tumour necrosis factor alpha (TNFα) or

interleukin 10 (IL) whereas CD56^{dim} NK cells have a slightly reduced capability to produce and release cytokines (reviewed in Cooper et al. 2001).

NK cells arise from CD34^{+} hematopoietic progenitors in the bone marrow and differentiation into NK cells is mainly triggered by the transcription factors Tbet and Eomes. In the periphery NK cells differentiate from CD56^{bright} via CD56^{dim} to CD56^{negative} NK cells accompanied by a decline in telomere length (Romagnani et al. 2007). Furthermore, Björkström et al. (2010) described additional differentiation stages within the CD56^{dim} NK cell population. CD56^{bright} NK cells express high amounts of NKG2A whereas CD56^{dim} NK cells continuously lose NKG2A while they steadily gain the expression of CD57 and killer cell immunoglobulin-like receptors (KIRs).

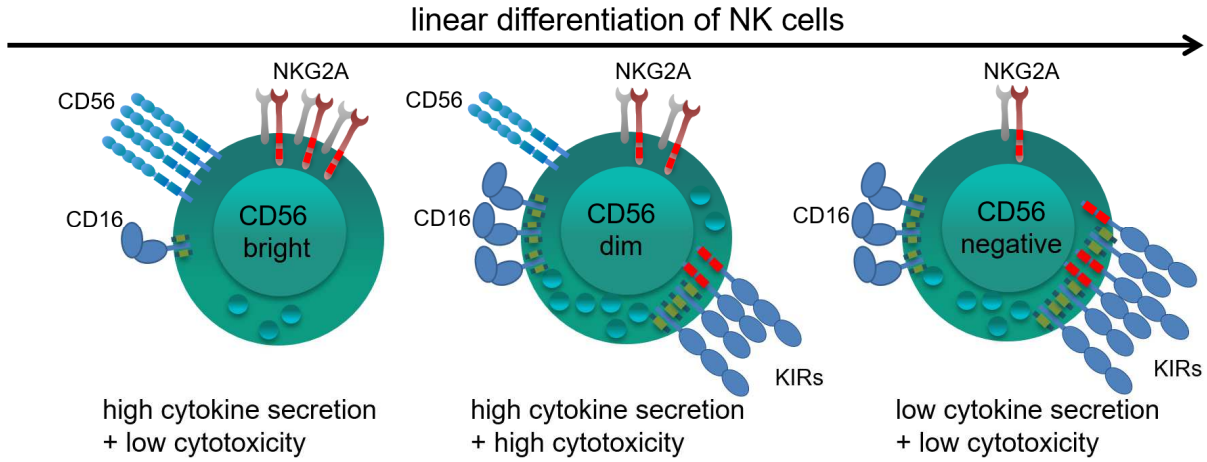

Figure 1.2: NK cell differentiation

NK cells differentiate from CD56^{bright} to CD56^{dim} to CD56^{negative} NK cells. Differentiation is accompanied by the subsequent loss of CD56 and NKG2A and the acquisition of CD16 and killer cell immunoglobulin-like receptors (KIRs). NK cell subsets are associated with different functional properties. CD56^{bright} NK cells produce a wide range of cytokines, whereas their ability to produce cytotoxic granules is restricted. In contrast, CD56^{dim} NK cells are highly capable to release cytolytic granules and are efficient cytokine producers, although to a lower extent than CD56^{bright} NK cells. CD56^{-} NK cells display impaired functionality and proliferation potential.

Early reports suggested mature NK cells to be terminally differentiated, incapable of self-renewal and short lived (Miller 1982, Zhang et al. 2007). However, in an adoptive transfer experiment into a lymphopenic environment NK cells underwent homeostatic proliferation, resulting in longevity of transferred NK cells residing in both lymphoid and non-lymphoid tissue for over six months. NK cells are capable of self-renewal at a slow turnover rate, thus display similarities with T cells. Moreover, NK cells retain their functional capacity and respond robustly to virus infections even months after transfer (Sun et al. 2011). Even though NK cells are considered members of the innate immunity, over the last years various features of NK cells have been described

that were initially assigned exclusively to adaptive immune cells. For instance, NK cells are educated during development (reviewed in Orr et al. 2010), NK cells undergo clonal expansion during infection and they are able to generate long lived memory NK cells as seen in cytomegalovirus (CMV) infection (Sun et al. 2009).

1.2.1 NK cell education

To gain the capacity to detect target cells with downregulated or low MHC class I surface expression, NK cells must undergo a process called education which is also referred to as licencing or tuning of NK cells. Whereas during T cell education an activation via the TCR is required, the NK cell education process is based on the engagement of MHC-class I specific inhibitory receptors leading to the maturation of a functional NK cell repertoire (reviewed in Höglund et al. 2010, and Orr et al. 2010). The extent of NK cell responsiveness is determined by the number of interactions of inhibitory receptors with self MHC class I molecules on haematopoietic or stromal cells during development in the bone marrow. In contrast to the negative selection process during T cell development, NK cells that are not able to engage MHC class I through inhibitory receptors are not driven into apoptosis but become anergic (Kim et al. 2005, Yu et al. 2007). Notably, the NK cell education process is not terminal and NK cells are capable of readjustment. Anergic NK cells placed in a different MHC-class I setting acquire functional competence, whereas educated NK cells become hyporesponsive in a MHC-class I molecules devoid environment. To maintain their responsiveness NK cells need the continuous engagement of their inhibitory receptors to MHC-class I molecules (Joncker et al. 2010).

1.2.2 NK cell activation and effector mechanisms

NK cells express various receptors including inhibitory, activating, adhesion and cytokine receptors enabling them to differentiate between healthy and target cells. In a healthy setting NK cells remain in a tolerant state. Healthy cells express MHC class I molecules that are engaged by inhibitory receptors on NK cells balancing activating and inhibiting signals. Loss or downregulation of MHC class I molecules, seen on tumour or virus infected cells, however, leads to an activation of NK cells due to missing self-recognition. Absence of MHC class I engagement results in cytokine production and release of cytotoxic granules of NK cells (reviewed in Ljunggren et al. 1990). Furthermore, activation of NK cells through activating

receptors such as NKG2D can be induced by upregulation of activating ligands like MICA as a result of cellular stress (figure 1.3) (Bauer et al. 1999).

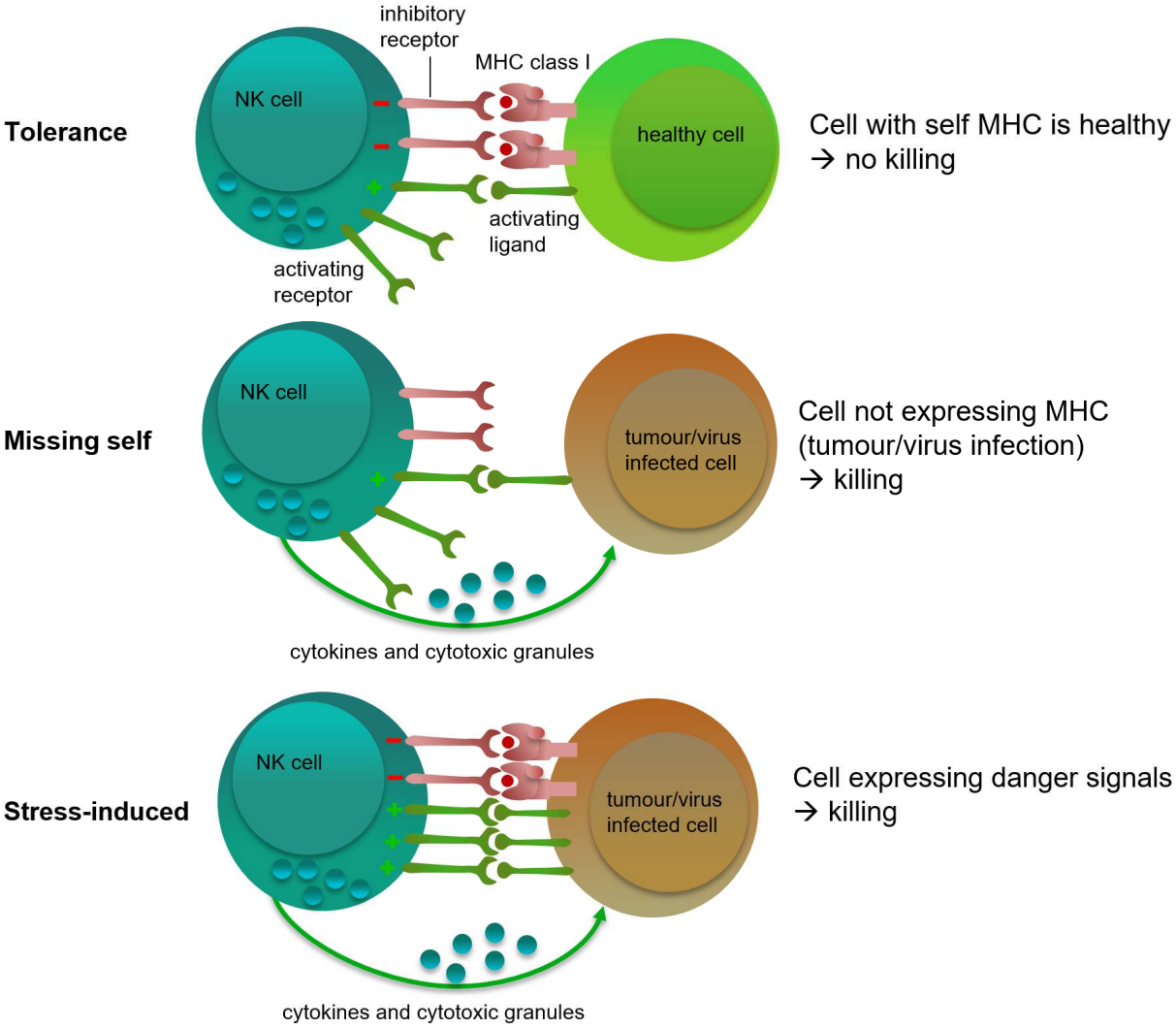

Figure 1.3: NK cell activation by tumour or virus infected cells

NK cells remain in a tolerant state when the strength of the activating signal received through engagement of activating receptors is lower than the inhibitory signal through binding of inhibitory receptors to MHC-class I molecules. Virus infected and tumour cells either downregulate or loose the expression of MHC-class I molecules on the cell surface and therefore, NK cells lack the inhibitory signal. The activating signal is not dampened resulting in missing-self-induced cytokine production and release of cytotoxic granules by NK cells. Another mechanism to activate NK cells to produce cytokines and cytotoxic granules is stress-induced. Stressed cells upregulate activating ligands, thus the activation signal outweighs the inhibitory signal (reviewed in Vivier et al. 2012).

As described, NK cells are able to recognise stressed cells triggering various effector mechanisms with the aim of eliminating the target cell. NK cell activation leads to the production of cytokines including IFNγ and TNFα. Moreover, target cells can be lysed by release of cytotoxic granules containing perforin or granzyme. Apoptosis of target cells can be induced via surface upregulation of FasL as well as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) on NK cells and binding to Fas as well as TRAIL receptors on the target cell surface. Furthermore, NK cells mediate

cellular crosstalk by stimulating dendritic cell (DC) maturation and therefore enhancing priming of T cells. Stressed cells which are opsonised by antibodies trigger ADCC of NK cells. The activating receptor CD16, also called FcγRIII, can recognise those cell bound antibodies and through crosslinking leads to direct killing of opsonised cells by triggering the release of cytotoxic granules (figure 1.4) (reviewed in Parham 2009, and Cheent et al. 2011)

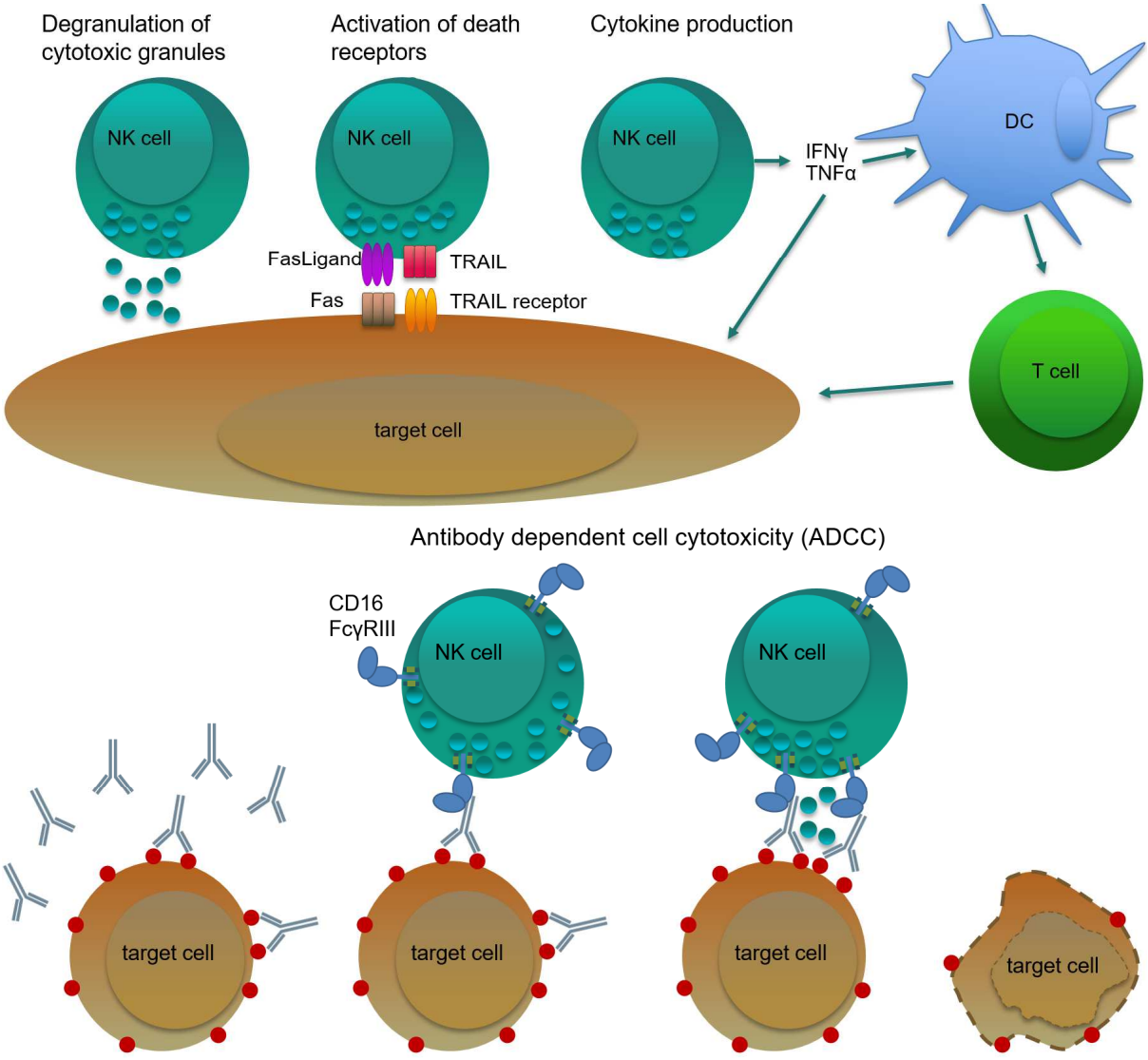

Figure 1.4: NK cell effector mechanisms

NK cells are able to release cytotoxic granules resulting in direct cytotoxic effects to target cells. Moreover, they can induce apoptosis via surface upregulation of Fas ligand (FasL) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Through the engagement with Fas and TRAIL receptors on the target cell surface, NK cells can directly kill tumour or virus infected cells. Furthermore, NK cells secrete IFNγ and TNFα which mediate a direct antiviral effect and induce adaptive immune responses (A) (reviewed in Cheent et al. 2011).The activating receptor CD16, also called FcγRIII, can generate ADCC. CD16 recognises cells which are opsonised by antibodies and directly kill them through the release of cytotoxic granules after crosslinking of CD16 with the antibodies (B) (reviewed in Parham 2009).

1.2.3 NK cell receptors

NK cells express a variety of activating and inhibitory receptors on the cell surface mediating their activation and inhibition. These NK receptors engage MHC class I molecules, MHC class I–like molecules, and molecules unrelated to MHC on the target cell. In the human system, we can group most NK cell receptors into three receptor families: the killer cell immunoglobulin-like receptor (KIR) family, natural cytotoxicity receptors (NCRs) and the CD94/NKG2 receptor family (reviewed in Caligiuri 2008).

1.2.4 Killer cell immunoglobulin-like receptors (KIRs)

The first identified KIRs were inhibitory receptors leading to the initial naming as killer cell inhibitory receptors. Further studies determined that inhibitory as well as activating receptors belong to this receptor family therefore KIR was kept as the abbreviation for killer cell immunoglobulin-like receptors (Marsh et al. 2003). KIRs are able to bind to HLA class I molecules like HLA-A, -B, -C, –G and -F and to date 14 expressed human KIR genes and two KIR pseudogenes have been described (Gonzalez-Galarza et al. 2015, Garcia-Beltran et al. 2016). KIRs are encoded on chromosome 19q13.4 in a compact cluster of genes that is part of the leucocyte receptor complex. Nomenclature of KIRs is mainly based on their protein structure with subdivisions according to the number of extracellular domains and the length of the cytoplasmic tail. The KIRs have either two (2D) or three (3D) extracellular immunoglobulin (Ig) domains and either a short (S) or a long (L) cytoplasmic tail (figure 1.5). Furthermore, there is a less frequently used naming according to the cluster of differentiation (CD) nomenclature system classifying KIR molecules as members of the CD158 series. The length of the cytoplasmic domain determines if KIRs signal through an immunoreceptor tyrosine-based inhibition motif (ITIM) or an immunoreceptor tyrosine-based activation motif (ITAM). KIRs expressing a long cytoplasmic domain transmit inhibitory signals through ITIMs located in their cytoplasmic domains (Carrington et al. 2003 pages 8-11) whereas, KIRs with a short cytoplasmic tail transmit activating signals through crosslinking of KIR with DAP12. DAP12 is an homodimeric adapter molecule which contains ITAMs in its cytoplasmic domain. Crosslinking of KIR and DAP12 leads to phosphorylation of DAP12 and therefore to downstream signalling through binding of ZAP-70 and Syk protein tyrosine kinases (Lanier et al. 1998).

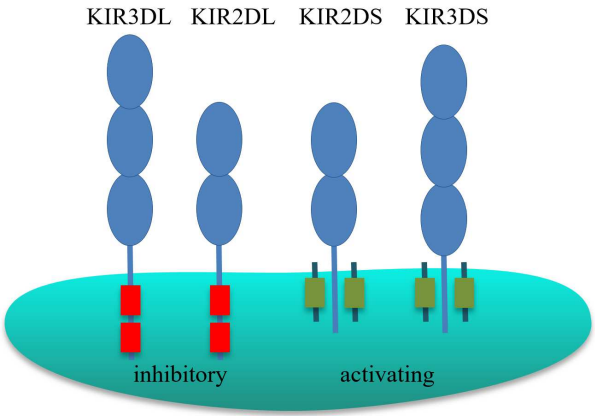

Figure 1.5: KIR nomenclature

The nomenclature of KIRs is based on the number of extracellular immunoglobulin (Ig)-like domains dividing them into KIR2D having two domains and KIR3D comprising three extracellular domains. Irrespective of the number of Ig domains KIRs either have a long or a short cytoplasmic tail appearing as (L) or (S) in the KIR nomenclature. While inhibitory KIRs have a long cytoplasmic tail and signal through a ITIM motif (red) activating KIRs have a short cytoplasmic tail and signal trough the association with an adapter protein DAP-12 containing an ITAM motif (green).

For most KIR genes allelic variations have been described and their nomenclature is analogous to the one used for HLA alleles. An asterisk separates the gene name from the first three digits indicating the alleles that differ in the sequence of the encoded protein. Another four digits are used to discriminate alleles either having silent substitutions in the coding region or substitutions in an intron, promoter, or other noncoding regions of the sequence (Marsh et al. 2003). Based on the nomenclature KIR3DL1*001 for instance, is the first allele of the inhibitory KIR3DL1 receptor with a long cytoplasmic tail and three extracellular Ig domains (Gonzalez-Galarza et al. 2015).

1.2.5 KIR3DL1

KIR3DL1 shows a high allelic variability with 79 alleles described until now (Gonzalez-Galarza et al. 2015). According to the cell surface density, alleles can be grouped into alleles associated with intracellular retention and no cell surface expression (null) as well as alleles showing either low or high KIR3DL1 levels. In contrast to other pairs of inhibitory and activating receptors, KIR3DL1 and KIR3DS1 are encoded in the same locus and segregate as alleles. The ligand for KIR3DL1 is a Bw4 motif comprised in a polymorphic region at amino acid positions 77–83 of HLA-A and HLA-B molecules. This region defines HLA alleles that are not capable to bind KIR3DL1 containing a so called Bw6 motif or alleles with a Bw4 motif able to interact with KIR3DL1. Amino acid positions 78 and 79 are conserved between the Bw4 and Bw6 motifs. The HLA class I residues that distinguish the Bw4 and Bw6 motif from

one another are amino acids 77, 80, 81, 82 and 83 as depicted in table 1.1 (Gumperz et al. 1997).

Table 1.1: HLA class I residues determining Bw4 and Bw6 motifs

	polymorphic position within HLA class I molecules				
epitope	77	80	81	82	83
Bw4	N/D/S	I/T	A/L	L	R
Bw6	S/G	N	L	R	G

A: alanine, R: arginine, D: aspartic acid, N: asparagine, G: glycine, I: isoleucine, L: leucin, S: serine, T: threonine

The binding affinity of KIR3DL1 to the HLA molecule is determined by a dimorphic residue at position 80 of the Bw4 motif. HLA-Bw4 alleles with isoleucine (80I) are stronger ligands than alleles with threonine (80T). Furthermore, KIR3DL1 is described to bind not only to the Bw4 motif itself but also to the presented peptide. Particularly, positions 7 and 8 of the presented peptide are crucial for the interaction with KIR3DL1 (reviewed in Parham et al. 2012, and O'Connor et al. 2013). In contrast to KIR3DL1, binding assays could not identify a ligand for KIR3DS1 until 2016. Genetic as well as functional studies suggested that HLA-Bw4 80(I) is a potential ligand (Martin et al. 2002, Alter et al. 2007) but recent evidence demonstrated the binding of KIR3DS1 to HLA-F and therefore identifying the ligand for KIR3DS1(Garcia-Beltran et al. 2016).

1.2.6 NK cells in HCV infection

Since NK cells are highly enriched in the liver comprising up to 50% of the total lymphocyte population and the fact that HCV is a hepatotropic infection, many studies focused on the role of NK cells in HCV infection. Reports suggest NK cells to be activated in the acute phase of HCV infection but without apparent correlation with HCV clearance. This activation is characterised by an enhanced IFNγ production and a higher cytotoxicity compared to healthy individuals (Amadei et al. 2010, Pelletier et al. 2010). In addition, the expression of NK cell receptors is associated with HCV infection outcome. Alter et al. (2011) reported that low frequencies of $NKp30^+$, $NKp46^+$, $CD161^+$, and $NKG2D^+$ NK cells during acute infection correlate with spontaneous resolution of HCV infection. NK cells during acute infection are mostly associated with high functional competence, whereas impaired NK cell functionality has been described in chronic infection. IFNα leads to a functional polarisation of NK cells towards high cytotoxicity and impaired IFNγ production in chronic infections. Persistent exposure to endogenous IFNα modulates NK cell responsiveness to

become refractory correlating with the loss of efficacy of IFNα based antiviral therapies (Edlich et al. 2012). Highly activated NK cells in chronic infection with enhanced cytolytic activity and dysfunctional cytokine production correlate with the degree of liver inflammation (Oliviero et al. 2009). Moreover, NK cells in chronic HCV infection are associated with reduced NKp46 and NKp30 expression and a decrease in NCR mediated killing of target cells (Nattermann et al. 2006) even though contradicting reports suggest elevated NCR expression during chronic infection (De Maria et al. 2007, Ahlenstiel et al. 2010). Furthermore, NK cells can contribute to immune protection prior to seroconversion as described in studies of high-risk groups such as PWID or healthcare workers exposed to HCV due to needlestick injuries. Seronegative individuals display a higher proportion of effector $CD56^{low}$ mature NK cells associated with increased cytolytic activity. Moreover, expression of the activating receptor NKp30 protects against HCV infection in a high-risk cohort of PWID (Golden-Mason et al. 2010). In addition, healthcare workers that have been percutaneously exposed to HCV without seroconversion show increased NK cell responses like IFNγ production and cytotoxicity as well as enhanced expression of NKp44, NKp46 and NKG2A (Werner et al. 2013). Furthermore, the presence of $KIR2DL3^{+}$ $NKG2A^{-}$ NK cells is associated with protection from seroconversion in HCV exposed individuals (Thöns et al. 2014). Collectively these data show that NK cells contribute to an anti-HCV negative state in HCV exposed individuals therefore providing protection from HCV infection prior to seroconversion.

1.2.7 KIRs and infection

Genetic association studies have associated certain KIR/KIR-ligand combinations with viral infection outcome. Patients harbouring the genetic combination of KIR2DL3 with two HLA-C group1 (C1) alleles are more likely to spontaneously clear the infection than heterozygous C1/C2 or homozygous C2/C2 individuals (Khakoo et al. 2004). Moreover, the same genetic combination has been correlated with protection from HCV infection prior to seroconversion in a PWID cohort. Furthermore, individuals with this genetic constellation are more likely to achieve SVR if undergoing treatment (Knapp et al. 2010). In addition, the genetic constellation of KIR3DL1 with Bw4 is associated with increased SVR in genotype 1b infected patients treated with PEGylated IFNα and ribavirin in combination with or without telaprevir (Umemura et al. 2014).

1.3 Natural Killer T (NKT) cells

Clearance of HCV infection has been associated with the activation of the innate immune system as well as the subsequent activation of the adaptive immune system. 20 years ago, a new cell subset has been identified forming a link between both. Natural Killer T cells (NKT cells) were first described in the mid-1990s as a subset of T cells in mice that share some characteristics with NK cells, specifically the expression of the NK cell marker NK1.1. The characterisation of NKT cells as a T cell subset was based on the expression of a TCR (Lantz et al. 1994, Makino et al. 1995). To date NKT cells are grouped as an intermediate between the innate and the adaptive immune system since they share characteristics of T cells as well as NK cells (reviewed in Taniguchi et al. 2003). NKT cells can be subdivided into two groups; Type I NKT cells, also termed invariant NKT (iNKT) cells and Type II NKT cells also referred to as non-invariant NKT cells. iNKT cells are defined by a highly conserved semi-invariant TCR. In mice, the TCR is encoded by Vα14 and Jα18 paired with a set of Vß chains like Vß8.2, Vß7 and Vß2 (Lantz et al. 1994). In contrast, the human TCR receptor comprises a Vα24Jα18 chain exclusively combined with Vß11 (Dellabona et al. 1994). Non-invariant NKT cells in mice as well as in humans express a more diverse TCR repertoire (Behar et al. 1999). In comparison to conventional T cells that recognise antigens presented by MHC molecules, NKT cells recognise antigens presented by the non-classical MHC class I like molecule CD1d. CD1d is essential for the presentation of glycolipids to NKT cells. The first identified glycolipid ligand for iNKT cells was α-Galactosylceramide (αGalCer), an exogenous lipid isolated from the marine sponge *Agalas mauritianus* (Kawano et al. 1997). Although invariant as well as non-invariant NKT cells are CD1d restricted, non-invariant NKT cells do not recognise α-linked glycolipids presented through CD1d, thus are not αGalCer responsive. Besides this prototypic antigen for iNKT cells, various other glycolipid antigens have been described for iNKT cells. These include bacterial lipids like mycobacterial phosphatidylinositol mannoside or endogenous glycolipids such as the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) (Fischer et al. 2004). The endogenous iGb3 is recognised by mice as well as human iNKT cells and is implicated in mediating iNKT cell development. Moreover, iGb3 expression in the peripheral tissue is involved in controlling iNKT cell responses for instance to infections (Zhou et al. 2004).

Initially, NKT cells were defined by the expression of NK1.1 in mice and CD161 in humans. However, CD161 negative NKT cells have been reported in the periphery suggesting that the detection and definition of NKT cells requires adaptation (Pellicci et al. 2002). Since αGalCer has been identified as a strong antigen presented by CD1d, multimers were designed to identify αGalCer-CD1d restricted iNKT cells. These multimers of αGalCer loaded CD1d are highly sensitive and specific for identifying invariant Vα14$^+$ NKT cells in mice. CD1d restricted non-invariant NKT cells, however, do not recognise and respond to αGalCer presentation, thus are not detected with this method. Therefore, αGalCer loaded CD1d multimers have been proposed as an effective tool to study CD1d restricted iNKT cells in mice (Matsuda et al. 2000).

1.3.1 NKT cell differentiation

NKT cells develop in the thymus similar to T cells. The highest frequency of NKT cells can be detected early in the fetal age and the numbers decrease continuously with age. Human NKT cells are able to leave the thymus at an undifferentiated stage to further differentiate in the periphery (Sandberg et al. 2004). Like classical T cells, NKT cells undergo a positive selection process. Therefore, immature double positive cortical thymocytes (CD4$^+$CD8$^+$) present CD1d the selecting ligand for NKT cell development. This positive selection is crucial to ensure, that the TCR of NKT cells can interact with CD1d (Bendelac 1995). As previously described, NKT cells are often defined by CD161 and the maturation process of NKT cells is associated with an increase of CD161 expression. However, most NKT cells leave the thymus at an immature CD161$^-$ state and continue their maturation process in the periphery. Therefore, CD161$^-$ cells represent precursors of more mature NKT cells in the periphery (Pellicci et al. 2002). In the thymus, the majority of NKT cells are CD4$^+$ whereas in the periphery NKT cells can acquire the expression of CD8, thus based on their expression of CD4 and CD8 four different NKT cell subsets can be defined in the periphery (Baev et al. 2004, Montoya et al. 2007). Nevertheless, there is only a limited correlation between NKT cell frequencies in the thymus and in the peripheral blood (Berzins et al. 2005). The main transcription factor for the development of nonconventional T cells, including NKT cells is the promyelocytic leukaemia zinc finger protein (PLZF). PLZF leads to the development of fully functional NKT cells which are capable of secreting cytokines like IFNγ and IL4 as well as being able to release cytotoxic granules (Kovalovsky et al. 2008). In addition, histone

deacetylase 3 has recently been identified as being essential for NKT cell development and NKT cell differentiation into functional NKT cells (Thapa et al. 2017).

1.3.2 CD1d restricted activation of invariant NKT (iNKT) cells

The TCR of NKT cells from mice and humans recognises the antigen presenting molecule CD1d. Even though mice CD1 and human CD1d only exhibit a sequence similarity of approximately 60% in the antigen binding region, the interaction of the invariant TCR with CD1 is highly conserved between both species. The hydrophobic antigen binding groove enables CD1d to present lipid antigens, more specifically glycolipids in most cases. Mouse as well as human CD1d can present the iNKT prototype antigen αGalCer leading to a rapid activation of iNKT cells (Brossay et al. 1998). CD1d is able to present self-ligands as well as foreign lipid antigens that can activate iNKT cells. The binding affinity of endogenous lipids like iGb3 is low in comparison to αGalCer. However, the induction of a weak TCR signal by low affinity antigens can be sufficient to induce iNKT cell activation if a second signal by inflammatory cytokines like IL12 is provided. Such a strong cytokine signal can for instance, be represented by IL12 that is produced in response to microbial products by antigen presenting cells like DCs. This way of signalling is proposed to be the main mechanism of iNKT cell activation in microbial and viral infections (Brigl et al. 2003) (figure 1.6).

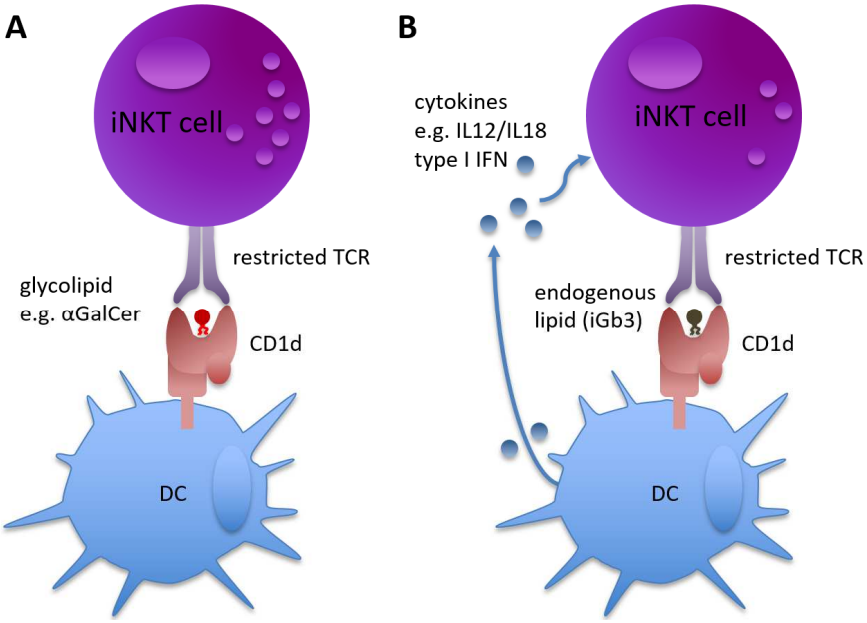

Figure 1.6: Activation of iNKT cells

iNKT cell activation is dependent on two signalling mechanisms: a TCR signal, provided by the engagement with a glycolipid-CD1d complex and a cytokine driven signal. iNKT cells can be activated through a TCR signal provided by the presentation of a strong foreign antigen as αGalCer that is largely independent of further cytokine stimuli (A). In contrast, a weak TCR signal by binding to low affinity microbial or self-lipids as iGb3, requires a strong cytokine signal. Activation can be mediated by the release of pro-inflammatory cytokines like IL12 from antigen presenting cells (B).

1.3.3 CD1d the ligand for NKT cells

The human CD1 family comprises five CD1 genes (CD1a, b, c, d, and e) located on chromosome 1, whereas mice only express the equivalent to CD1d. CD1 proteins are mainly expressed on cells that are involved in antigen presentation, such as the majority of thymocytes, DCs, macrophages, monocytes or B cells (reviewed in Porcelli et al. 1999). High CD1d expression was detected on hepatocytes whereas MHC class I expression in the liver was poor. These data indicate that CD1d restricted cells such as iNKT cells are important regulators of immune responses in the liver (Agrati et al. 2005)

1.3.4 iNKT cell effector mechanisms

iNKT cells are cytokine producing effector cells that also mediate direct cytolysis by release of cytotoxic granules. The main priming mechanism for the cytolytic activity of the $CD4^-$ iNKT cell subset, is the exposure to cytokines like IL2 and IL12. Moreover, this subset primarily secretes T helper (TH) 1 cytokines such as IL2, IFNγ and TNFα. $CD4^+$ CD1d restricted iNKT cells, however, are mainly characterised by their production of TH2 cytokines like IL4, even though they are able to produce TH1 cytokines as well. In addition, they can mediate cytolysis by releasing cytotoxic

granules containing perforin, in comparison to $CD4^-$ iNKT cells, however, this cytolytic ability is not mediated by cytokines (Gumperz et al. 2002).

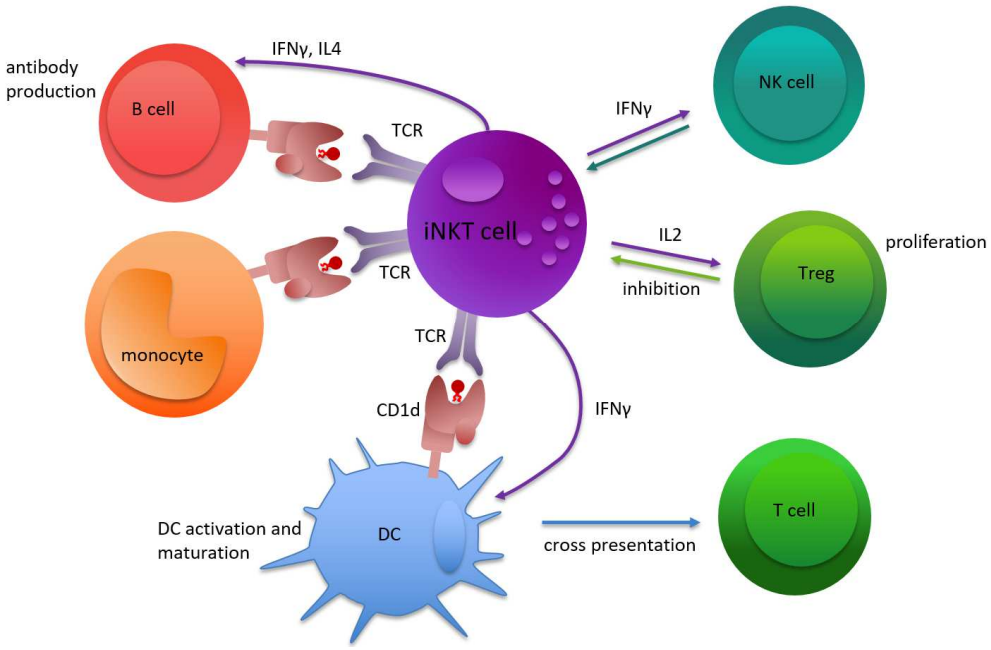

Figure 1.7: iNKT cell crosstalk with the innate and adaptive immune system

iNKT cells can regulate and activate various cells of the innate and adaptive immune system. NKT cell activation can lead to DC maturation and increased antibody production of B cells. Furthermore, NK cells can be induced by iNKT cells to produce IFNγ. Treg proliferation is enhanced by IL2 secretion of NKT cells and naïve T cells are indirectly regulated through cross presentation by DCs.

IFNγ produced by iNKT cells can directly activate NK cells. Activated NK cells are driven to produce IFNγ and therefore, enhance the induction of IFNγ production further, suggesting a prompt activation of the innate immune system by iNKT cells (Carnaud et al. 1999). NKT cells have the capability to recognise myeloid DCs early during the immune response through their CD1d specific TCR. NKT cells induce maturation of DCs and activate them to produce IL12. For this activation induced IL12 production, a second signal besides the TCR signal is needed and provided by CD40L engagement (Vincent et al. 2002). Thus, by NKT cell mediated maturation of DCs, the adaptive immunity is modulated by the increased potential of mature DCs to efficiently stimulate the proliferation of naïve T cells. Furthermore, iNKT cells can provide signals for B cell activation by interaction with CD40 on the B cell surface. iNKT cells are therefore able to improve antibody titers and B cell memory responses (Galli et al. 2007). In addition, IL2 produced by NKT cells can induce the development of regulatory T cells (Treg), whereas these in turn inhibit NKT cell proliferation, cytokine release and the production of cytotoxic granules (figure 1.7) (reviewed in La Cava et al. 2006).

1.3.5 iNKT cells in disease

In different disease settings iNKT cells mediate promoting or inhibitory effects. CD1d restricted iNKT cells, especially their production of IL4, is associated with protection from type I diabetes. In type I diabetic patients a strong bias towards TH1 cytokines like IFNγ and a loss in the capability of iNKT cells to secrete IL4 has been described (Wilson et al. 1998). Moreover, iNKT cells can be beneficial in microbial and fungal infections. Activation of iNKT cells confers protection from Mycobacterium tuberculosis and IFNγ production by activated iNKT cells leads to local resistance to the fungi Cryptococcus neoformans (Kawakami et al. 2001, Chackerian et al. 2002). In a mouse model iNKT cell activation has been shown to inhibit Hepatitis B virus (HBV) replication (Kakimi et al. 2000) postulating, that activated intrahepatic iNKT cells are important during natural infection and contribute to viral clearance. Clearance of HBV deoxyribonucleic acid (DNA) in the blood as well as in the liver is correlated with an early increase in IFNγ levels and is not mediated by the adaptive immune system (Guidotti et al. 1999). Therefore, IFNγ production can be either attributed to NK or iNKT cells or perhaps an interplay of both.

1.3.6 iNKT cells in HCV infection

Several studies have determined iNKT cell frequencies in the periphery as well as in the liver of HCV infected individuals. However, reports are highly inconsistent. Some reports suggest no difference in frequencies whereas, others state that decreased frequencies are associated with chronic infection and increased frequencies with a seronegative state. Healthcare workers that have been exposed to HCV infection without seroconversion show increased frequencies of CD1d multimer$^+$ iNKT cells six weeks after exposure accompanied by an increase in NKG2D expression on iNKT cells (Werner et al. 2013). Inoue et al. (2006) and van der Vliet et al. (2005) do not observe any differences in frequency of Vα24$^+$ Vβ11$^+$ iNKT cells in the periphery between chronically HCV infected patients and healthy individuals. Whereas, significantly lower frequencies of Vα24$^+$ Vβ11$^+$ iNKT cells in HCV-RNA positive individuals in comparison to patients that resolved the infection and healthy controls have been described by Lucas et al. (2003). Furthermore, Vα24$^+$ iNKT cells are depleted in the liver of individuals with cirrhotic chronic HCV infection (Deignan et al. 2002).

1.4 DNA methylation

NK cells can confer early immune control or prevention of HCV infection by their cytolytic activity resulting in killing of virus infected hepatocytes or priming of the adaptive T cell immunity (Golden-Mason et al. 2010, Pelletier et al. 2010). NK cell differentiation, essential for the development of responsive NK cells as well as their effector functions, for instance, the capacity to produce IFNγ is epigenetically regulated. Epigenetic modifications include posttranslational histone modifications, modifications of the DNA and chromatin modulating processes that are crucial for the regulation of various cellular processes comprising the expression of genes and microRNA, X-chromosome inactivation or cellular differentiation. Modifications like methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, deimination and proline isomerisation can alter the gene activity without affecting the underlying DNA sequence (reviewed in Kouzarides 2007). DNA methylation is the most intensely studied modification and the occurrence of hypomethylation was first described in 1983 in human cancer. Feinberg et al. (1983) showed that cancer cells harbour unmethylated CpG clusters whereas CpG sites within normal tissues are methylated.

DNA methylation occurs at cytosine residues that are located in CpG dinucleotides. The process of the addition of a methyl group transferred from S-adenosylmethionine to the fifth carbon of the cytosine forming a five-methyl-cytosine (5mC) is catalysed by a DNA methyltransferase. These CpG sites cluster in regions called CpG islands commonly present in gene promoters or in gene regulatory regions but being rare within genes. DNA methylation is associated with decreased or inhibited gene expression due to interference of the 5mC with the binding of transcription factors therefore, preventing the formation of transcription factor complexes required for optimal transcription (Watt et al. 1988). Moreover, gene transcription can be repressed by the binding of methyl-CpG binding domain proteins to 5mC, thus blocking transcription factor binding. In contrast, hypomethylation of CpG islands leads to gene activation and transcription (reviewed in Portela et al. 2010). For mapping DNA methylation patterns, bisulfite genome sequencing is a reliable method for the detection of methylated cytosine. Upon treatment of the DNA with bisulfite, cytosines are deaminated and therefore converted into uracil. However, methylated cytosine is not influenced by this treatment and therefore remains unchanged. This

allows discrimination after amplification by polymerase chain reaction (PCR) and subsequent sequencing of the region of interest (Frommer et al. 1992).

1.4.1 DNA methylation of NK cells

In early NK cell maturation stages KIR genes are epigenetically silenced. During the NK cell differentiation process KIRs become transcribed through opening of chromatin structures and demethylation of the promoter region. Furthermore, KIR3DL1 promoter methylation determines the allele-specific expression on the NK cell surface. The promoter of KIR3DL1$^-$ NK cells is highly methylated, whereas KIR3DL1$^+$ NK cells display a completely unmethylated promoter (Chan et al. 2003). However, only a few studies focused on the functional impact of DNA methylation on NK cells. Long term activation of NK cells is associated with a methylation profile similar to activated lymphocytes especially T cells. Hypomethylation and therefore activation has been described in the loci of TNFA family members and the TH2 cytokine IL13. Activated NK cells display high demethylation, whereas naïve NK cells are mostly methylated suggesting the NK cell methylome might display plasticity which can be remodelled during activation (Wiencke et al. 2016).

1.4.2 DNA methylation and IFNγ competence of immune cells

The functional capacity of immune cells to express IFNγ is correlated with the DNA methylation status. Hypomethylation within the *IFNG* promoter is associated with high IFNγ capacity. Complete methylation of CpG sites within the *IFNG* gene and within a transcriptional activator element preceding the *IFNG* gene is seen in naïve CD4$^+$ T cells and neonatal T cells that are associated with low IFNγ competence, whereas CD8$^+$ T cells and effector CD4$^+$ T cells with high IFNγ competence show hypomethylation in the *IFNG* locus. The differentiation process of naïve CD4 T cells to TH1 cells that is accompanied by epigenetic alterations within the *IFNG* locus is needed to gain the capability to efficiently produce IFNγ (Melvin et al. 1995). Distal cis-regulatory elements in proximity to the *IFNG* gene are described to be associated with initiation of *IFNG* transcription through recruitment of transcription factors (Balasubramani et al. 2010). Therefore, methylation of distal regions impacts IFNγ production by immune cells. Similar to T cells changing their epigenetic profile during differentiation from naïve to TH1, NK cells undergo epigenetic changes of the *IFNG* promoter during their differentiation process. The maturation process from CD56^{bright} NK cells to CD56^{dim} NK cells is associated with demethylation of the *IFNG* promoter

and a proposed acquisition of a higher IFNγ competence (Luetke-Eversloh et al. 2014).

1.5 Aims of the study

The most important risk factor in western industrialised countries to acquire HCV infection is intravenous drug use. The high infection rates among PWID are caused by their common practice of sharing needles and other injection materials. For this reason, we utilise a PWID cohort to study HCV infection and comparatively analyse PWID developing chronic infection, spontaneously resolving HCV infection and PWID remaining anti-HCV seronegative. The aim is to provide novel insight into innate and adaptive mechanisms of the immune system leading to spontaneous immune control or the protection against HCV infection prior to seroconversion. Since the role of iNKT cells for the outcome of HCV infection is poorly defined and contradicting reports regarding described frequencies in chronic HCV infected individuals exist, the aim is to comparatively analyse frequencies and function of iNKT cells in HCV-RNA positive and anti-HCV positive PWID with resolved HCV infection. iNKT cells are still poorly covered by scientific reports in HCV infection, however, there is growing evidence for the importance of NK cells for HCV infection outcome. Different genetic association studies revealed that distinct KIR/KIR ligand constellations are associated with differential outcome in infections. Therefore, the aim is to analyse the impact of different KIR/KIR-ligand genotypes on the outcome of HCV infection in PWID. Moreover, KIR/KIR ligand constellations might influence NK cell phenotype and function which will be addressed in this study. Epigenetic modifications can alter gene expression and therefore influence cytokine competence of various immune cells including NK cells. Since IFNγ production of NK cells is a crucial effector mechanism for the outcome of HCV infection and DNA methylation in the *IFNG* region impacts IFNγ production the aim is to analyse if NK cells with different KIR/KIR ligand constellations are differentially regulated by epigenetic modifications.

1. The role of iNKT cells in HCV infection
    a. Is there a distinct iNKT cell phenotype associated with HCV infection outcome?
    b. Do iNKT cells differ in their functionality depending on the HCV status?
2. Association of KIR/KIR-ligand constellations on HCV infection outcome in our PWID cohort
    a. Do genetically determined KIR/KIR ligand constellations that correlate with infection outcome influence the phenotype and function of NK cells?
3. Epigenetic regulation of the IFNγ competence
    a. Is the IFNγ production of NK cells with different KIR/KIR ligand combinations associated with distinct methylation patterns in the *IFNG* locus?

2 Materials

2.1 Chemicals and reagents

α-Galactosylceramide (αGalCer)	Funakoshi
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (BD CompBeads)	Becton Dickinson (BD)
BD FACS™Clean	BD
BD FACS™Flow	BD
BD FACS™ Shutdown Solution	BD
BD FACS™Rinse	BD
Biozym LE Agarose	Biozym
Brefeldin A (BFA)	Sigma
Cellclean® CL50	Sysmex
Cellpack®	Sysmex
CellTrics® filter 50 µm	Sysmex
Cytometer Setup & Tracking Beads Kit (CST beads)	BD
Biocoll Separating Solution	Biochrome
dimethyl sulfoxide (DMSO) ≥99.8%	Roth
DNA ladder 100 bp 0.5 mg/mL	peQlab
DNA loading Dye 6x	Thermo Scientific
dNTP mix 10 mM each	Invitrogen
Dulbecco's Phosphate-Buffered Saline, 1X (PBS)	Gibco
Ethanol absolute	Merck
Ethidium bromide solution 0.025% 250 µg/mL	Roth
GeneRuler 1kb DNA ladder 0.5 µg/µl	Thermo Scientific
IC Fixation Buffer	eBioscience (eBio)
Interferon alpha A, alpha2a, human	Pbl assay science
Interleukin-28B/ Interferon λ3, human	Pbl assay science
IL12 Human	Reprokine
IL15 Human	Reprokine
IL18 Human	Reprokine
Ionomycin	Calbiochem
Nuclease-Free water	Ambion
PCR Buffer 10x 15 mM $MgCl_2$	Qiagen
Permiabilization Buffer (10x)	eBio

Platinum® Taq DNA polymerase	Invitrogen
PMA	Sigma
2-Propanol	VWR
Stromatolyser®-WH	Sysmex
SYBR® Green PCR Master Mix	Applied biosystems
Tris-Borate-EDTA buffer	Sigma

2.2 Cell culture media and additives

Fetal bovine serum (FBS) superior	Biochrome
Interleukin2 (IL2) human	Roche
HEPES Buffer Solution (1M)	Gibco
Penicillin/ Streptomycin (100x) PenStrep	Gibco
RPMI Medium 1640 1x + GlutaMAX™	Gibco

2.3 Composition of cell culture media

Prior to supplementation of Fetal bovine serum (FBS) superior to the medium it was heated for 30 min at 56°C to inactivate the complement system.

R10	RPMI Medium 1640 10% FBS 10 mM HEPES 100 IU/mL penicillin 100 µg/mL streptomycin
Freezing medium	FBS 10% DMSO

2.4 Cell line

The K562 cell line is a human erythroleukemic cell line that is highly undifferentiated. It was established by Lozzio et al. (1975) from a pleural effusion of a 53-year-old woman with a chronic myelogenous leukaemia. In NK cell assays K562 cells are highly sensitive targets for killing as they are HLA devoid.

2.5 Commercial kits

EZ DNA Methylation-Direct™ Kit	Zymo Research
LIVE/DEAD® Fixable Blue Dead Cells Stain Kit	Life technologies
QIAamp® DNA Blood Mini Kit	Qiagen
QIAquick® PCR purification Kit	Qiagen

2.6 Antibodies for flow cytometry

All conjugated antibodies were stored at 4°C and were mouse anti-human if not stated otherwise. Table 2.1 indicates the amount of antibody that is needed to stain $1x10^6$ cells.

Table 2.1: Antibodies for flow cytometry

specificity	fluorochrome	clone	isotype	µl/10^6 cells	company
αGalCer loaded CD1d dextramer	allophycocyanin (APC)	n.a.	n.a.	5	immudex
CD1d	peridinin chlorophyll (PerCP)-eFluor™710	51.1	IgG2b	5	eBio
CD3	APC-eFluor® 780	OKT3	IgG2a	2	eBio
CD3	eFluor® 450	OKT3	IgG2a	2	eBio
CD3	PerCP-Cy5.5	OKT3	IgG2a	1	eBio
CD4	APC-eFluor® 780	RPA-T4	IgG1	2	eBio
CD4	phycoerythrin (PE)	RPA-T4	IgG1	1	eBio
CD8a	APC	RPA-T8	IgG1	1	eBio
CD8a	eFluor® 450	RPA-T8	IgG1	0.2	eBio
CD14	PE-Cy7	61D3	IgG1	1	eBio
CD14	PerCP-Cy5.5	61D3	IgG1	2	eBio
CD14	APC-eFluor® 780	61D3	IgG1	2	eBio
CD16	APC-eFluor® 780	eBioCB16	IgG1	1	eBio
CD16	PE	B73.1	IgG1	1	eBio
CD19	APC-eFluor® 780	HIB19	IgG1	1	eBio
CD19	PerCP-Cy5.5	SJ25C1	IgG1	2	eBio
CD38	PerCP-eFluor™ 710	HB7	IgG1	1	eBio
CD45RA	PE-Cy7	HI100	IgG2b	0.5	eBio
CD45RO	Fluorescein isothiocyanate (FITC)	UCHL1	IgG2a	2	eBio
CD56	Brilliant Violet (BV) 421™	HCD56	IgG1	2	Biolegend
CD56	FITC	MEM188	IgG2a	5	eBio
CD56	PerCP-eFluor™ 710	CMSSB	IgG1	0.5	eBio
CD57	FITC	TB01	IgM	0.5	eBio
CD107a	PE-Cy7	H4A3	IgG1	2	BD

specificity	fluorochrome	clone	isotype	µl/10^6 cells	company
CD127	PE-Cy7	HIL-7R-M21	IgG1	5	BD
CD158b2/KIR2DL3	FITC	180701	IgG2A	5	R&D
CD158e1/KIR3DL1	APC	DX9	IgG1	2	Biolegend
CD159a/ NKG2A	PE-Cy7	Z199	IgG2b	5	Beckman Coulter (BEC)
CD159c/ NKG2C	PE	134591	IgG1	5	R&D
CD161	PE-Cy7	HP-3G10	IgG1	5	eBio
CD197/CCR7	PE	3D12	Rat/ IgG2a	5	eBio
CD272/BTLA	PE	MIH26	IgG2a	2	Biolegend
CD278/PD1	BV421™	EH12.2H7	IgG1	2	Biolegend
CD314/NKG2D	PerCP-eFluor™ 710	1D11	IgG1	1	eBio
Fixable Viability Dye	eFluor™ 506	n.a.	n.a.	0.1	eBio
IFNγ	FITC	4S.B3	IgG1	1	eBio
IL2	PerCP-eFluor™ 710	MQ1-17H12	Rat/ IgG2a	2	eBio
TNFα	PE	MAb11	IgG1	1	eBio
Vα24	FITC	C15	IgG1	5	BEC
Vβ11	PE	C21	IgG2a	5	BEC

n.a.: not applicable

2.7 Oligonucleotides

All primers were ordered from Eurofins. Lyophilised primers were reconstituted in nuclease-free water at a concentration of 10 pmol/µl and stored at -20°C. Table 2.2 depicts all primers used for the DNA methylation analysis and the estimated PCR product size.

Table 2.2: Primer sequences

Primer name	region	Primer sequence 5´ - 3`	product size
IFNγ forward IFNγ reverse	IFNγ promoter	TTGAATGGTGTGAAGTAAAAGTG (23 bp) CAACCACAAACAARTACTATTAAAAA (26 bp)	543 bp
CNS1 forward CNS1 reverse	CNS1 region	AGAAAAGGGGGGATTTA (17 bp) TAACACTCACAACCAAATTATC (22 bp)	247 bp

2.8 Patients

Blood samples from healthy individuals were collected from the centre for blood donation from the University Hospital Düsseldorf with approval of the local ethics

committee. Due to an anonymisation process after the collection no donor information concerning age and gender was available.

Whole blood samples from treatment-naïve patients with a history of intravenous drug use were collected from the ward for inpatient detoxification treatment of drug addicts or the clinic for opioid maintenance treatment at the Department for Addiction Medicine and Addictive Behaviour of the LVR-Hospital Essen, Hospital of the University of Duisburg-Essen. Written informed consent was obtained from all patients included in this study. The study was approved by the ethics committee of the Medical Faculty of the University of Duisburg-Essen. Samples were tested for anti-HCV antibodies by a chemiluminescent microparticle immunoassay from Abbott. Presence of HCV-RNA was determined by Abott RealTime HCV PCR assay with a detection limit of 12 IU/mL. PWID were grouped according to their anti-HCV and HCV-RNA status into three groups: 1. anti-HCV seropositive with detectable HCV-RNA (HCV-RNA positive), 2. anti-HCV seropositive without detectable HCV-RNA (HCV-RNA negative) and 3. Anti-HCV seronegative PWID without detectable HCV-RNA (anti-HCV negative).

In addition, blood samples of anti-HCV positive PWID from North America were collected at the infectious diseases or hepatology clinics at Massachusetts General Hospital in Boston with local ethics committee approval. Data from these patients were kindly provided by Georg M. Lauer and Arthur Y. Kim.

2.9 Consumables and equipment

Allegra® X-15R Centrifuge	BEC
Cell culture flask 25 cm^2, 75 cm^2	Corning
Cell culture flask 175 cm^2	Thermo Scientific
Cell culture plate (48 well)	Greiner
Cell culture plate (24 well, 96 well)	Corning
Cell scraper	TPP
Cellstar® tubes 15 mL, 50 mL(Falcon)	Greiner
Centrifuge 5415 D	Eppendorf
Centrifuge 5810R	Eppendorf
Combitips advanced® 5 mL, 10 mL	Eppendorf
Cryo tubes, 2.0 mL	Greiner Bio-One
FACS Canto II	BD

Filter tips 10 µl, 10/20 µl, 20 µl, 200 µl, 1000 µl	Starlab
Freezer (-20°C)	Bosch
Agagel mini/maxi gel electrophoresis chamber	Biometra
Ice machine AF100	Scotsman
Incubator 37°C BBD 6220	Heraeus
Laminar flow Herasafe	Thermo Scientific
Leucosep™ tubes 50 mL	Greiner
Liquid nitrogen tank Biosafe® MD	Cryotherm
Liquid nitrogen tank HEco Series 800-190	MVE
Mastercycler nexus GSX1	Eppendorf
Megafuge 40R	Thermo Scientific
MicroAmp® Optical 8-cap strip	Applied Biosystems
Microwave	Küppersbusch
Microscope Primovert	Zeiss
Microscope TS100 eclipse	Nikon
MoFloXDP	BEC
Mr. Frosty™ Cryo 1°C freezing Container	Nalgene
Multipette® plus	Eppendorf
Multichannel 8 research 300 µl	Eppendorf
Multichannel 12 research plus 30-300 µl	Eppendorf
NanoDrop 2000	Thermo scientific
Sprout Minicentrifuge, PCR tube centrifuge	Biozym
Syringe filters 0.8 µm	Whatman
Syringe Omnifix® 20 ml	Braun
PCR Tube Strips 0.2 mL	Eppendorf
Pipettes research tips (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Eppendorf
pipetus®	Hirschmann
Polystyrene Round-Bottom FACS tubes 5 mL	BD
7500 Real time PCR System	Applied Biosystems
Reagent reservoir 50 mL	Corning
Refrigerator	Bosch
Safe-Lock tube 2 mL	eppendorf
Safe Seal tube 1.5 mL	sarstedt
Scale JL602-G	Mettler Toledo

Scale ScoutPro	Ohaus
Stripette® 2 mL, 5 mL, 10 mL, 25 mL	costar
Thermomixer comfort	Eppendorf
Thermocycler T3000	Biometra
Thermocycler Professional TRIO	Biometra
ThermoStat plus	eppendorf
Tissue culture plate (6 well, 12 well)	TPP
Tissue culture plate 96 well-U	VWR
96 well U bottom tissue culture plate	BD
Ultra-Low temperature freezer (-80°C) U725-G Innova®	New Brunswick
UV-System	Intas
Vortexer VV3	VWR
Vortexer L46	Labinco
Water bath	Köttermann
XP-300 cell counter	Sysmex

2.10 Software and webpages

Allele Frequency Net Database	http://www.allelefrequencies.net
FlowJo 10.0.7	Tree Star Inc.
Geneious 7.1.9	Biomatters Ltd.
Graph Pad Prism 5.04	GraphPad Software, Inc.
Microsoft Office 2010	Microsoft Corporation

3 Methods

3.1 Standard cell culture methods

3.1.1 Thawing of cells

Frozen cells (PBMCs or K562) were taken out of the liquid nitrogen and quickly thawed in a 37°C water bath. The cells were transferred into 15 mL tubes containing 9 mL of prewarmed PBS. Afterwards the cells were centrifuged at 754 x *g* for 7 min. The supernatant was discarded and the pellet resuspended in 10 mL of PBS. Following another washing step, the cells were counted with the XP-300 cell counter and resuspended in the appropriate amount of media or PBS depending on the assay. Cells were either transferred into cell culture flasks or plates for further culture whereas cells for *ex vivo* fluorescence-activated cell sorting (FACS) analysis were transferred into 96 well U bottom tissue culture plates or polystyrene round-bottom FACS tubes for subsequent staining.

3.1.2 Freezing of cells

Cells were centrifuged at 754 x *g* for 7 min and the pellet was washed once with PBS. Afterwards the cell pellet was resuspended in 500 µl of FBS and transferred into a 2 mL cryo vial containing 500 µl FBS with 20% dimethyl sulfoxide (DMSO), thus cells were frozen in a final concentration of 10% DMSO in FBS. Cells were frozen slowly at a rate of approximately 1°C per minute in a Mr. Frosty™ freezing container, containing 2-Propanol and placed at -80°C to ensure optimal cell viability. Thereafter cells were transferred into the liquid nitrogen tank.

3.1.3 Splitting of cells

Cells were maintained in culture at 37°C, 5% CO_2 and 95% humidified atmosphere. Cells lines were regularly checked for their confluence and passaged appropriately. Therefore, suspension cells were resuspended thoroughly and transferred into a new flask containing fresh media. Cell debris was removed whenever necessary by centrifugation at 149 x *g* for 7 min. Moreover, cells lines were regularly checked for mycoplasma contaminations (described in 3.4.5) to ensure that all experiments were performed with mycoplasma free cells.

3.2 Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated whole blood from PWID or buffy coats from healthy individuals via Ficoll density centrifugation (Boyum 1968). The isolation of mononuclear cells is based on different migration properties of peripheral cells during centrifugation. Centrifugation leads to the formation of several layers containing different cell types. While the bottom layer contains by Biocoll aggregated erythrocytes the intermediate layer contains mostly granulocytes. The mononuclear cell layer is visible as a small white ring in between the ficoll and the plasma layer. The interphase above the filter contains cells which are not dense enough to pass the Biocoll layer and consists of platelets, monocytes and lymphocytes.

To obtain plasma, for instance, for the analysis of the CMV serostatus anticoagulated blood was centrifuged for 15 min at 1455 x *g*. Plasma was stored at -80°C until further analysis. For the isolation of PBMCs, blood was diluted 1:2 with PBS to ensure an optimal separation. 35 mL of blood-PBS were pipetted slowly into a 50 mL Leucosep™ tube containing 15 mL of Biocoll Separating Solution beneath the filter. After a 10 min centrifugation at 1126 x *g* the interphase, containing the PBMCs was transferred into a 50 mL tube and centrifuged at 754 x *g* for 7 min. To ensure removal of residual Biocoll and platelets, cells were washed twice in a total volume of 50 mL with PBS. Prior to the second washing step 200 µl cell suspension were transferred in a 1.5 mL tube and frozen at -20°C for subsequent DNA isolation (described in 3.4.1). Furthermore, the cell number was determined with the XP-300 cell counter and the cell pellet was resuspended in freezing medium. Cell aliquots were transferred into a Mr. Frosty™ freezing container and placed in a -80°C freezer. After at least 80 min cryo vials were placed in the liquid nitrogen.

3.3 Cultivation of PBMCs

PBMCs were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% Pen/Strep (10 000 IU/mL Penicillin, 10 000 µg/mL Streptomycin) and 1% HEPES Buffer (1 M) at 37°C, 5% CO_2 and 95% humidity.

3.4 Standard molecular biological techniques

3.4.1 Isolation of DNA from PBMCs

Genomic DNA was extracted from frozen PBMCs (aliquoted during 3.2) using the QIAamp® DNA Blood Mini Kit. Isolation was performed according to the manufacturer's instructions. In short, PBMCs were thawed and 20 µl of QIAGEN protease was added to the cell suspension. Following the addition of 200 µl of AL buffer the mixture was homogenised by pulse-vortexing for 15 s. For efficient lysis, the mixture was incubated for 10 min at 56°C. After the incubation, 200 µl of ethanol were added and the sample was transferred to a QIAamp Mini Spin Column. Following a centrifugation for 1 min at 5939 x *g* the flow through was discarded and 500 µl of washing buffer AW1 were added. Following another centrifugation, the spin column was transferred into a new 2 mL collection tube and washed with 500 µl of buffer AW2 at 15682 x *g* for 3 min. Thus, the DNA bound to the QIAamp membrane, was washed twice with two different washing buffers which resulted in improved purity of subsequently eluted DNA and ensured removal of contaminants. In order to remove all remaining AW2 buffer, the column was placed in a new collection tube and centrifuged again for 1 min at 15682 x *g*. To elute the purified DNA the spin column was placed into a 1.5 mL tube. Subsequently 50 µl of nuclease-free water were added and after a 1 min incubation the DNA was eluted by centrifugation for 1 min at 5939 x *g*. To increase DNA yields the elution step was repeated. Therefore, the purified DNA was eluted in a final volume of 100 µl. The DNA concentration was determined afterwards via measurement with the NanoDrop 2000.

3.4.2 HLA typing

DNA from patients and healthy individuals was isolated as described in 3.4.1. HLA class I typing at four-digit resolution-level (HLA-A, HLA-B and HLA-C) was performed by the use of sequence-specific oligonucleotides (LABType methodology) provided by One Lambda Inc. (Canoga Park, CA), at the Institute for Transfusion Medicine, University Hospital Essen.

3.4.3 KIR typing

DNA from patients was isolated from PBMCs using Qiagen spin columns (3.4.1) and KIR genotyping was performed as previously described (Thöns et al. 2014). In short,

by use of sequence-specific oligonucleotides KIR typing was performed on a Luminex™ flow analyser platform at the Institute for Transfusion Medicine University Hospital Essen. Consistency of genotypes was verified through comparison with all published KIR haplotypes on Allele Frequency Net (Gonzalez-Galarza et al. 2015). DNA based KIR3DL1 allele subtyping was performed as described by Boudreau et al. (2014) with adjusted cycling conditions at the Institute for Transplantation Diagnostics and Cell Therapeutics, University Hospital Düsseldorf. KIR3DL1 subgroups were determined according to the subtyping in null, low or high.

3.4.4 Measurement of CMV IgG antibodies

CMV serostatus was determined in the routine diagnostic lab of our institute via a quantitative detection of CMV specific IgG antibodies, based on a chemiluminescent immunoassay method. Therefore, plasma was tested with the LIAISON® CMV IgG II assay and analysed on a LIAISON® XL.

3.4.5 Detection of mycoplasma in cell cultures

Cell cultures were regularly checked for mycoplasma contamination. Therefore, cell culture supernatants of 3-day old cultures were analysed via an inhouse Real-time-Sybr-Green PCR.

3.5 Flow cytometric analysis

Performance checks via measurement of CS&T beads were regularly performed to ensure an optimal flow cytometer setup of the BD FACS Canto II. Through the measurement of single stained cells or BD CompBeads a compensation for each individual panel was calculated. BD CompBeads were utilised for panels that included rare cell populations or dimly expressed cell surface antigens whereas cells were used for panels that comprised only highly expressed antigens.

3.6 Flow cytometric identification of iNKT cells

In order to determine iNKT cells through flow cytometry, $3x10^6$ PBMCs were stained with antibodies against the invariant T cell receptor and a commercially available fluorescently labelled CD1d dextramer loaded with αGalCer. Therefore, PBMCs were thawed and transferred to polystyrene round-bottom FACS tubes. Cells were stained for 20 min with the APC labelled CD1d dextramer at room temperature (RT) in a total volume of 100 µl in PBS. After incubation cells were washed with 2 mL of PBS and centrifuged for 5 min at 524 x *g*. The supernatant was removed by decantation.

Afterwards cells were stained with 100 µl of fixable viability dye eFluor506 for 15 min at 4°C to distinguish between viable and dead cells in the subsequent analysis. Following another washing step, the cells were stained with fluorochrome-conjugated antibodies against the invariant TCR chains Vα24 (FITC) and Vβ11 (PE). Furthermore, the cell surface antigens CD3 (eFluor450) and CD19 (APC-eFluor 780) were stained for 15 min at 4°C. After cell surface staining, cells were washed and live cells were fixated at 4°C for 15 min by the addition of 100 µl of cold IC fixation buffer. Treatment with IC fixation buffer results in the fixation of cells through cross-linking of proteins. Fixated cells were washed with PBS and acquired on a BD FACS Canto II. During analysis with FlowJo 10.0.7 $CD3^+$ iNKT cells were defined after the exclusion of $CD19^+$ cells due to unspecific binding of the CD1d dextramer to B cells.

3.7 Phenotypical characterisation of iNKT by flow cytometry

To determine possible differences in the phenotype of iNKT cells from HCV-RNA positive and HCV-RNA negative individuals, PBMCs were quickly thawed and the iNKT cell frequency was determined through αGalCer loaded CD1d dextramer staining. Therefore, $2x10^6$ PBMCs were transferred into polystyrene round-bottom FACS tubes and stained for 20 min with the CD1d dextramer (APC) at RT in a total volume of 100 µl in PBS. Afterwards the cells were washed with 2 mL of PBS and centrifuged at 524 x *g* for 5 min. Following the staining of dead cells with fixable viability dye eFluor506 prediluted in 100 µl of PBS, for 15 min at 4°C, cells were washed again with 2 mL of PBS. PBMCs were stained for the cell surface antigen CD19 (APC-eFluor 780) for 15 min at 4°C. After washing with PBS and fixation with 100 µl of IC fixation buffer for 15 min at 4°C, cells were acquired on a BD FACS Canto II. For phenotypic *ex vivo* analysis of iNKT cells, $3x10^6$-$5x10^6$ PBMCs were stained in two separate steps with the αGalCer loaded CD1d dextramer (APC) and the viability dye as previously indicated. Subsequently PBMCs were stained for various cell surface antigens with additional fluorochrome-conjugated antibodies. All panels included CD19 (APC-eFluor 780) as a marker to exclude $CD19^+$ B cells. A master mix containing all antibodies of interest was prepared for each individual panel and staining was performed in a total volume of 100 µl. PBMCs were stained for CD38 (PerCP-eFluor 710), CD127 (PE-Cy7), CD161 (PE-Cy7), CD57 (FITC), NKG2A (PE-Cy7), NKG2D (PerCP-eFluor 710), KIR2DL3 (FITC), PD1 (BV421) and BTLA (PE). After an incubation time of 15 min at 4°C cells were washed with PBS

and fixed with 100 µl of IC fixation buffer. Prior to flow cytometric measurement on the BD FACS Canto II cells were washed once again with PBS.

3.8 Expansion of iNKT cells with αGalCer

PBMCs were thawed and resuspended at a concentration of $2x10^6$ cells/mL in R10. The medium was supplemented with 25 IU/mL of recombinant IL2. Afterwards PBMCs were stimulated with the exogenous glycolipid αGalCer (1 µg/mL) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 5-7 days half of the total volume R10 containing 25 IU/mL recombinant IL2 was added to the culture. To determine the expansion potential of iNKT cells and possible changes in the activation status $1x10^6$ cells were stained after 10 days of expansion with the αGalCer loaded CD1d dextramer (APC) and antibodies against the cell surface antigens CD38 (PerCP-eFluor 710) and CD127 (PE-Cy7) after 10 days of expansion as stated in 3.7. To evaluate possible changes to the initial situation, *ex vivo* staining was performed as well.

3.9 Analysis of iNKT cell function via intracellular cytokine staining

iNKT cell function was analysed *ex vivo* and after *in vitro* expansion. On day 10 after stimulation with αGalCer $5x10^5$-$1x10^6$ cells were transferred into polystyrene round-bottom FACS tubes whereas *ex vivo* analysis was performed with $2x10^6$ PBMCs. Cells were washed twice with R10 medium at 524 x *g* for 5 min and then stimulated with 1 µg/mL Ionomycin and 10 ng/mL Phorbol myristate acetate (PMA) for 5 hours in the presence of Brefeldin A (BFA) (100 ng/mL). For determining the degranulation ability an antibody against CD107a (PE-Cy7) was directly added to the culture. As a negative control, one well remains unstimulated without the addition of PMA and Ionomycin. During the 5 hour incubation period BFA disrupts the structure and function of the Golgi apparatus and therefore prevents the transport of vesicles from the endoplasmic reticulum to the cell surface. Intracellular vesicles are retained in the cell allowing a detection of produced IFNγ and IL2 during the incubation time. Afterwards PBMCs were washed with 2 mL of PBS and pelleted at 524 x *g* for 5 min. Following another washing step with 2 mL PBS cells were stained with a APC labelled αGalCer loaded CD1d dextramer for 20 min at RT in the dark. Following a washing step, cell viability was determined through staining with a fixable viability dye eFluor506 for 15 min at 4°C in a total volume of 100 µl to exclude dead cells. Cells were washed with 2 mL of PBS at 5 min at 542 x *g*. Afterwards PBMCs were stained

with antibodies against the surface antigens CD4 (PE), CD8 (eFluor450) and CD19 (APC-eFluor 780) in a total volume of 100 µl for 15 min at 4°C. Cells were washed once with PBS and PBMCs were fixed for 15 min at 4°C with 100 µl of IC fixation buffer. Cells were permeabilised by two washing steps with 1x Permeabilization Buffer. Through permeabilisation small holes were created in the cell membrane that allow intracellular staining of cytokines. The cells were stained for intracellular IFNγ (FITC) and IL2 (PerCP-eFluor 710) in 100 µl of 1x Permeabilization Buffer for 20 min at 4°C. Following another washing step with PBS the cells were resuspended in 100 µl PBS. Cells were acquired on a FACS Canto II and analysis was performed using FlowJo 10.0.7. The percentage of $IFN\gamma^+$, $IL2^+$, $CD107a^+$ iNKT cells was determined by subtracting the background cytokine production from the stimulated samples.

3.10 *In vitro* activation of iNKT cells

PBMCs from healthy individuals were stimulated either with IFNα or IFNλ3 or a combination of IL12, IL15 and IL18 to address the possible influence of these cytokines on the activation status of iNKT cells. The differences between the medium control without the addition of cytokines and the stimulated samples were determined. Changes in the activation status of iNKT cells was assessed through the measurement of the activation marker CD38 by flow cytometry. Therefore, PBMCs were quickly thawed and adjusted to a concentration of $2x10^6$ cells/mL in R10. After stimulation with IFNα (100 IU/mL) or IFNλ3 (1000 IU/mL) or IL12/15/18 (10 ng/mL, 100 ng/mL, 50 ng/mL) or IL12 (10ng/mL) or IL15 (100 ng/mL) or IL18 (100 ng/mL) over a period of 24h, $1x10^6$ cells were transferred into polystyrene round-bottom FACS tubes. Cells were washed twice with PBS to remove all stimulants. Following the αGalCer loaded dextramer staining (APC labelled), cells were stained with the fixable viability dye eFluor506 and subsequently with antibodies against the cell surface antigens CD19 (APC-eFluor 780) and CD38 (PerCP-eFluor 710). Prior to cell acquisition with the BD FACS Canto II cells were fixed.

3.11 CD1d expressing peripheral cells

To determine which cells in the periphery express CD1d on the cell surface, PBMCs from healthy individuals were analysed. Cell viability was evaluated by staining of dead cells via the fixable viability dye eFluor506. Following incubation, cells were washed and stained with antibodies against cell surface markers attributed to T cells,

B cells, NK cells and monocytes. While T cells, B cells and NK cells reside in the lymphocyte gate, monocytes display a different morphology and could be distinguished from the other cell types by analysing the forward and sideward scatter (FSC/SSC) plot. Cells were stained with antibodies targeting the cell surface antigens CD3 (eFluor 450), CD4 (PE) and CD8 (APC) to identify T cells and their subsets. Furthermore, NK cells were defined as $CD3^-/CD14^-/CD19^-$ and staining positive for CD56 (FITC) while B cells were identified as $CD19^+$ (APC-eFluor 780). In addition to determining monocytes by FSC and SSC they were identified after a 15 min incubation at 4°C with an antibody against CD14 (PE-Cy7). Following fixation and cell acquisition on a BD FACS Canto II cell surface staining for CD1d (PerCP-eFluor 710) was analysed for all cell types.

Furthermore, PBMCs from 13 HCV-RNA positive and 13 HCV-RNA negative PWID were analysed for their CD1d expression on monocytes and B cells. Therefore, $5x10^5$ cells were stained with the fixable viability dye eFluor506 to exclude dead cells. Subsequently cells were stained with antibodies against the cell surface antigens CD1d (PerCP-eFluor 710), CD14 (PE-Cy7) and CD16 (APC-eFluor 780) to define CD1d expressing monocytes. Whereas CD1d expressing B cells were determined after the addition of antibodies targeting CD19 (APC-eFluor 780) and CD1d (PerCP-eFluor 710) and subsequent incubation for 15 min at 4°C. Following cell fixation by IC fixation buffer cells were analysed by flow cytometry.

3.12 Influence of IFNα treatment on CD1d expression

PBMCs from healthy individuals were thawed and adjusted to a concentration of $2x10^6$ cells per mL in R10. Cells were treated with IFNα (100 IU/mL) or IFNλ3 (1000 IU/mL) or IL12/15/18 (10 ng/mL, 100 ng/mL, 50 ng/mL) over 24 h. As monocytes adhere to plastic cell surfaces, detachment prior to cell staining was performed. To avoid enzymatical digestion methods based for instance on trypsin that influences the cell viability, monocytes were detached by exposure to a cold environment. Following a centrifugation for 5 min at 754 x *g* the R10 was carefully removed. Ice cold PBS was added to the cells and the plate was placed at 4°C for 15 min to allow detachment. After incubation at 4°C, the remaining attached cells were detached by repeatedly pipetting up and down the PBS or cautious cell scraping. The level of detachment was visually analysed via an inverse microscope. $1x10^6$ stimulated cells as well as control cells, that were placed for 24 hours in R10 medium without further addition of stimulants, were transferred into polystyrene

round-bottom FACS tubes. Viable cells were determined through staining with the fixable viability dye eFluor506. Subsequently cells were washed with PBS and pelleted at 524 x *g* for 5 min. After discarding the supernatant, the cells were incubated with antibodies against CD14 (PE-Cy7), CD16 (APC-eFluor 780) and CD1d (PerCP-eFluor 710) for 15 min at 4°C. After an additional washing step cells were fixed and monocytes were analysed for their surface expression of CD1d by measurement on the BD FACS Canto II.

3.13 Monocyte depletion experiment

To investigate the role of monocytes, as a cell type expressing CD1d, in the expansion and activation of iNKT cells, PBMCs from 15 healthy individuals were expanded over a period of 10 days in the presence or absence of monocytes. Monocytes have the ability to adhere to plastic surfaces and this ability was used to deplete monocytes from the PBMC culture. PBMCs were adjusted to a concentration of $2x10^6$ cells per mL in R10. Cells were plated into two separate tissue culture plates, $5x10^6$ cells were transferred into a 12 well plate whereas $2x10^6$ cells were transferred into a 24 well plate. After 3 hours of incubation at 37°C monocytes were attached to the surface of the tissue culture plate. Non-adherent cells were carefully collected from the 12 well plate without scratching the adherent layer whereas the second plate remained untouched. Non-adherent cells were counted using a XP-300 cell counter and $2x10^6$ cells were transferred into a 24 well plate in a total volume of 1 mL R10. Subsequently untouched PBMCs as well as cells after monocyte depletion were stimulated with IL2 (25 IU/mL) and αGalCer (1 µg/mL). After 5 days of culture 500 µl R10 supplemented with IL2 (25 IU/mL) were added into each well. Frequency of iNKT cells as well as their expression of CD38 was analysed by flow cytometry directly after thawing and after 10 days of culture. For *ex vivo* analysis $2x10^6$ cells were used for staining while measurement of *in vitro* expanded cells were performed with $1x10^6$ cells. Cell viability (fixable viability dye eFluor506) and the expression of the cell surface antigens CD3 (eFluor 450), CD38 (PerCP-eFluor 710), CD19 (APC-eFluor 780) was determined on iNKT cells after staining with the αGalCer loaded CD1d dextramer (APC). Staining was performed as previously described in 3.7. Following cell fixation, cells were acquired on a BD FACS Canto II.

3.14 Isolation and analysis of intrahepatic lymphocytes

Liver tissue was obtained from patients undergoing liver resection at the University Hospital Essen. A perfusion of the resected liver piece was performed at the Department of Gastroenterology and Hepatology at the University Hospital Essen. Ruth Bröring kindly provided the liver perfusate comprising intrahepatic lymphocytes (IHL). The perfusate was transferred into 50 mL tubes and centrifuged at 524 x *g* for 7 min. After the supernatant was carefully decantated, the cell pellets were resuspended and combined. PBS was added up to a final volume of 35 mL. For IHL isolation 15 mL Bicoll were added into a 50 mL tube and the cell suspension was slowly layered over the Biocoll solution. Following a 20 min centrifugation at 1341 x *g* without brakes the mononuclear cell ring was transferred into a new tube. Subsequently cells were washed with PBS to remove residual Biocoll and pelleted at 524 x *g* for 7 min. Cells were resuspended in PBS and the cell number was determined. After another washing step cells were frozen in freezing media as described in 3.1.2. For determining intrahepatic iNKT cell frequencies, IHL were stained with the αGalCer loaded CD1d dextramer (APC) as previously described in chapter 3.7.

3.15 Flow cytometric analysis of KIR3DL1$^+$ NK cells

KIR3DL1$^+$ NK from 90 PWIDs either being HCV-RNA positive, HCV-RNA negative or anti-HCV negative and 120 healthy individuals were analysed for their IFNγ and TNFα secretion as well as CD107a expression. PBMCs were thawed and rested in R10 overnight at a concentration of $2x10^6$/mL. In addition, K562 were splitted 1:2 one-day prior to the stimulation assay to ensure optimal viability of the target cells. $5x10^5$ PBMCs were incubated with K562 at an effector target ratio of 1:10. As K562 are HLA devoid, engagement of inhibitory receptors on NK cells is abrogated, NK cells are activated and produce cytokines and cytotoxic granules. For the functional analysis of NK cells, PBMCs were washed once with R10 and $5x10^5$ PBMCs were transferred into a 96 well U bottom tissue culture plate. In parallel K562 cells, the NK cell targets, were counted and washed once with R10. After K562 cells were transferred, BFA (10 ng/mL) and a CD107a specific antibody (PE-Cy7) was added to the culture. Stimulation of NK cells with K562 cells was performed in a total volume of 250 µl of R10. For each analysed sample one well remained unstimulated, thus PBMCs were cultured in the presence of BFA without the addition of K562. After incubation at 37°C, 5% CO_2 and 95% humidity cells were pelleted during a 5 min

centrifugation at 524 x *g* and washed twice with PBS to remove all residual FBS, since proteins in the buffer reduce brightness of the viability staining. Staining for 15 min at 4°C with fixable viability dye eFluor506 allowed the exclusion of dead cells during subsequent analysis. Following another washing step PBMCs were stained with PerCP-Cy5.5 labelled antibodies against CD3, CD14 and CD19 to exclude T cells, monocytes and B cells. PBMCs were further stained with antibodies against the cell surface antigens CD16 (APC-eFluor 780), CD56 (BV-421) and KIR3DL1 (APC) to identify KIR3DL1 expressing NK cells. A master mix was prepared comprising all antibodies against cell surface antigens. Each sample was stained in a total volume of 100 µl of antibodies pre-diluted in PBS. Following an incubation at 4°C for 15 min, PBS was added and cells were centrifuged at 524 x *g* for 5 min. Afterwards cells were fixed through the addition of 100 µl IC fixation buffer for 15 min at 4°C. Subsequently cells were washed twice with 1x permeabilisation buffer and stained with antibodies against IFNγ (FITC) and TNFα (PE), previously intracellularly retained through the addition of BFA to the cell culture. Intracellular cytokine staining was performed in a total volume of 100 µl 1x permeabilisation buffer. Following incubation for 20 min at 4°C cells were washed with PBS and measured with a BD FACS Canto II. Subsequently results were analysed using FlowJo 10.0.7. and NK cell effector functions were calculated after the subtraction of the negative control.

3.16 Flow cytometric analysis of KIR3DL1$^+$ T cells

To determine the frequency of KIR3DL1 expressing T cells through flow cytometry, PBMCs from 15 healthy individuals were thawed and 5x10^5 PBMCs were transferred into a 96 well U bottom tissue culture plate. To exclude dead cells, a viability staining with fixable viability dye eFluor506 was performed. Subsequently cells were stained by antibodies against the cell surface antigens CD3 (PerCP-Cy5.5), CD4 (APC-eFluor 780), CD8 (eFluor450) and KIR3DL1 (APC). Following the 15 min incubation at 4°C, cells were washed and fixed by IC fixation buffer. Prior to the cell acquisition cells were washed once and the cell pellet was resuspended in PBS.

3.17 Flow cytometric analysis of NKG2C$^+$ NK cells

PBMCs from ten healthy individuals, that were included in the methylation analysis, were thawed and the NKG2C expression on NK cells was determined. 1x10^6 PBMCs were transferred into polystyrene round-bottom FACS tubes and stained for viable cells by the addition of fixable viability dye eFluor506. After an incubation for 15 min

at 4°C, cells were washed with 2 mL of PBS. Following a centrifugation for 5 min at 524 x *g* cells were stained with antibodies against the cell surface antigens CD3, CD14, CD19 (all PerCP-Cy5.5), CD16 (APC-eFluor 780), CD56 (BV-421), KIR3DL1 (APC) and NKG2C (PE).

3.18 CpG methylation analysis

To determine possible epigenetic imprinting leading to differences in IFNγ production, the methylation pattern of the *IFNG* region including the *IFNG* promoter and the conserved noncoding sequence (*CNS*)*1* region localised 4.2 kb upstream from the transcriptional start site of *IFNG* was analysed. $CD4^+$ T cells, TH1 cells and NK cells from healthy individuals were analysed for their DNA methylation pattern in the two selected regions.

3.19 Cell sort of naïve $CD4^+$ T cells, TH1 cells and NK cells for methylation analysis

The percentage of methylated CpGs of $CD4^+$ T cells, TH1 cells and NK cells was determined following FACS sorting of these cell populations. Therefore, frozen PBMCs from healthy individuals were thawed quickly in a 37°C water bath. The cells were transferred into 15 mL tubes containing 9 mL of prewarmed sterile filtered PBS. Afterwards the cells were centrifuged at 233 x *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 10 mL of sterile PBS. Following another washing step, cells were counted and $1x10^7$ cells were further used for staining. Cells were stained for 15 min at RT either with antibodies against the cell surface antigens CD3 (APC-eFluor 780) and CD56 (PerCP-eFluor 710) to define NK cells as $CD3^-$ $CD56^+$ or with CD3 (PerCP-Cy5.5), CD4 (APC-eFluor 780), CD45RA (PE-Cy7), CD45RO (FITC), CCR7 (PE) to investigate naïve CD4 T cells and TH1 cells. While both subsets express the T cell markers CD3 and CD4 they could be divided by their expression of CCR7, CD45RO as well as CD45RA into $CCR7^+$ $CD45RO^-$ $CD45RA^+$ naïve $CD4^+$ T cells and $CCR7^-$ $CD45RO^+$ $CD45RA^-$ TH1 cells. Subsequently cells were washed twice with sterile PBS and filtered prior to FACS based cell sorting using CellTrics® filters with a diameter of 50 µm. Cells were sorted through a BEC MoFlo XDP at the Core Flow Cytometry Facility of the University Hospital Düsseldorf.

3.20 Cell sort of $KIR3DL1^-$ and $KIR3DL1^+$ NK cells for methylation analysis

To determine the methylation pattern of KIR3DL1 negative and positive NK cells, frozen PBMCs were thawed as described in 3.19. PBMCs from ten healthy

individuals were stained in 500 µl sterile PBS for 15 min at RT using 0.5 µl/1x10^6 cells of live/dead fixable blue dead stain to distinguish viable from dead cells. Afterwards PBMCs were washed with 2 mL of sterile PBS and pelleted at 233 x *g* for 10 min. PBMCs were stained for surface antigens CD16 (PE), CD56 (PerCP-eFluor 710), KIR3DL1 (APC) and CD3/CD14/CD19 (all APC-eFluor 780) for 15 min at 4°C. The concentration of CD56 was adjusted to the detection with MoFlo XDP as the intensity of PerCP-eFluor 710 was dimmer on this cytometer, thus 2 µl instead of 0.5 µl were used per 1x10^6 cells, which allowed reliable detection of NK cells. Thereafter, cells were washed twice with 2 mL of sterile PBS and filtered before detection to avoid cell aggregates. Viable, single, lymphocytes which were $CD3^-$ $CD14^-$ $CD19^-$ and $CD56^{dim}$ NK cells were positively selected and further subdivided by their expression of KIR3DL1 into $KIR3DL1^-$ and $KIR3DL1^+$. Sorting was performed by a BEC MoFlo XDP.

3.21 Bisulfite treatment of DNA

Sorted global NK cells, naïve $CD4^+$ T cells, TH1 cells as well as $KIR3DL1^-$ and $KIR3DL1^+$ $CD56^{dim}$ NK cells were pelleted and dissolved in 12 µl of nuclease-free water. *Ex vivo* FACS sorted cells were treated with proteinase K and bisulfite converted by using the EZ DNA Methylation-Direct™ Kit according to the manufactures instruction. Through bisulfite treatment unmethylated cytosines were converted into uracil and could be detected as thymidin during sequencing while methylated cytosines were not affected by the treatment and remain as cytosine in the sequence (principle of bisulfite sequencing is depicted in figure 3.1). Sorted cells were digested with proteinase K. Therefore, 12 µl sample were mixed in a PCR tube with 13 µl digestion buffer (2x) and 1 µl proteinase K and incubated for 20 min at 50°C. Followed by a 5 min centrifugation at 9279 x *g*, 20 µl of the supernatant were used for the subsequent bisulfite conversion. The supernatant was mixed with 130 µl of CT Conversion Reagent and placed into the thermocycler. During 8 min at 98°C the proteinase K was inactivated and the DNA is thermally denaturated. During an incubation at 64°C for 3.5 hours the single stranded DNA was converted. Afterwards the sample could be stored up to 20 hours at 4°C. Following incubation 600 µl of M-binding buffer were added into a Zymo-Spin™ IC Column and the sample was mixed carefully with the buffer through inverting. While the converted single stranded DNA was bound to the membrane, centrifugation for 30 seconds at 15682 x *g* removed sodium bisulfite from the sample. After discarding the flow through the DNA

was washed with 100 µl of M-Wash Buffer to ensure complete removal of residual sodium bisulfite. The last step of the conversion process was the desulfonation into uracil by the addition of 200 µl of M-Desulphonation Buffer to the membrane bound DNA. The incubation period of 20 min at RT was followed by a centrifugation for 30 seconds at 15682 x *g*. Converted DNA was washed twice with 200 µl of M-Wash buffer before elution of the DNA by the direct addition of 10 µl M-Elution Buffer onto the membrane and subsequent centrifugation for 30 seconds at 15682 x *g*. Bisulfite converted DNA was stored at -80°C until further usage.

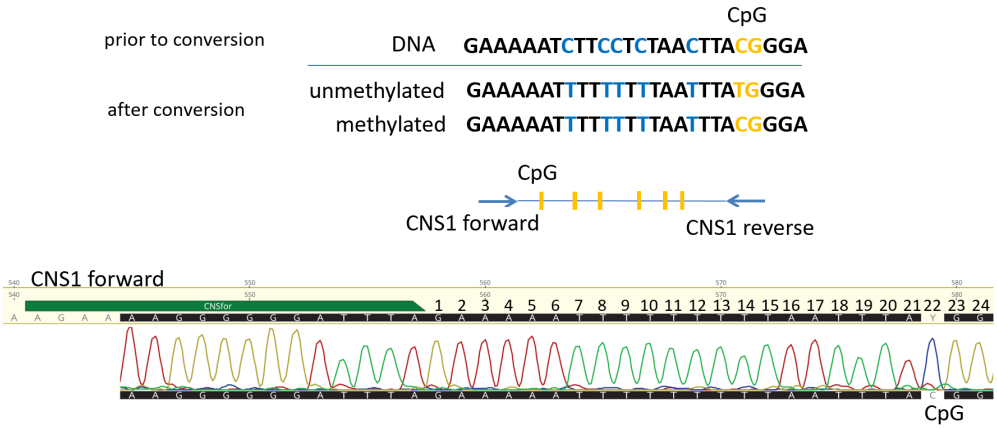

Figure 3.1: Principle of bisulfite sequencing

Bisulfite sequencing is a reliable method to distinguish methylated from unmethylated cytosines in CpGs of the region of interest. Unmethylated cytosines in a single stranded DNA are converted by bisulfite treatment into uracil and are detectable as thymine in the subsequent PCR amplification and sequencing (positions 8,11,12,14,18). However, methylated cytosines are not converted and remain as cytosine in the sequence (position 22). Therefore, through sequencing methylated CpGs can be distinguished from unmethylated CpGs and by calculating the ratio between cytosine and thymine the percentage of methylation can be determined. Furthermore, evaluation of cytosines outside the CpG sites can give insight into the efficacy of the bisulfite conversion, as they should be completely converted into thymine. The exemplary Sanger sequencing shown here provides proof of a complete conversion process and a high methylation of the analysed CpG. For quantitative instead of qualitative results from Sanger sequencing next generation sequencing was used to determine the frequency of methylation at each individual CpG site.

3.22 PCR amplification of bisulfite converted DNA

1 µl of bisulfite converted DNA was amplified using Platinum Taq DNA Polymerase and the composition of the PCR master mix is shown in table 3.3. The PCR reaction was performed in a total volume of 25 µl as the amplification protocol states (table 3.1 and table 3.2). Primer combinations used for the PCR are shown in table 2.2. Successful amplification of the bisulfite converted DNA was verified by gel electrophoresis. Therefore, 5 µl of PCR product were mixed with 1 µl of DNA loading dye (6x) and applied to a 1.5% agarose gel that comprised 5 drops of 0.025% ethidium bromide solution. The gel electrophoresis was performed in 1x Tris-Borate-EDTA buffer for 60 min at 120 V, 400 milliampere. The 247 bp (CNS1)

or 543 bp (IFNG promoter) sized PCR products were evaluated in comparison to the 100 bp and 1 kb base pair DNA ladder.

Table 3.1: PCR amplification protocol for the *CNS1* region

step	temperature	duration	cycles
initial denaturation	94°C	2 min	1
denaturation	94°C	0.5 min	45
annealing	52°C	0.5 min	
elongation	72°C	1 min	
final elongation	72°C	10.0 min	1

Table 3.2: PCR amplification protocol for the *IFNG* promoter

step	temperature	duration	cycles
initial denaturation	94°C	2 min	1
denaturation	94°C	0.5 min	45
annealing	50°C	0.5 min	
elongation	72°C	1 min	
final elongation	72°C	10.0 min	1

Table 3.3: PCR Master mix

component	volume
Nuclease-free water	19.9 µl
10 x Taq PCR Buffer (containing 15 mM $MgCL_2$)	2.5 µl
dNTP mix, 10 mM each	0.5 µl
Forward-primer 10 pmol/µl	0.5 µl
Reverse-primer 10 pmol/µl	0.5 µl
Platinum Taq DNA Polymerase	0.1 µl
Bisulfite treated DNA template	1 µl

3.23 PCR purification

After successful PCR amplification was confirmed, the PCR product was purified before sending it to Sanger sequencing. To remove impurities from the PCR product like primers, unused nucleotides and salts a PCR purification of amplified *CNS1* and *IFNG* products was performed according to the Qiagen PCR purification kit protocol. In short, 200 µl PB binding buffer and 20 µl PCR product was added into the spin column. Nucleic acids are able to bind to the silica membrane of the spin column. Therefore, all other ingredients could be discarded after a centrifugation for 30 seconds at 15682 x *g*. The DNA was washed with 750 µl PE buffer and the flow-through was discarded after 30 seconds of centrifugation at 15682 x *g*. An

additional centrifugation for 1 min was performed to remove residual ethanol comprised in the binding buffer. Afterwards the column was placed in a second 1.5 mL microcentrifuge tube and 30 µl EB buffer were added directly to the silica membrane. Followed by a 1 min incubation at RT the DNA was eluted through a centrifugation for 1 min at 15682 x *g*. The concentration of the purified DNA was measured with a Nano Drop at 260 nm since bisulfite converted DNA is A, U and T rich, the absorption resembles the absorption of RNA.

3.24 Sanger Sequencing

To confirm sufficient bisulfite conversion efficacy the purified DNA product was analysed by Sanger sequencing. Therefore, 50 ng of purified DNA in a total volume of 15 µl nuclease-free water was mixed with 2 µl (10 pmol/µl) of the specific primer for each of the analysed regions separately. The *IFNG* promoter region and the *CNS1* region were both sequenced with the forward as well as the reverse primer. The sequencing was performed by Eurofins and analysed with Geneious 7.1.9.

3.25 DNA methylation analysis of the *IFNG* locus by Next generation sequencing

After confirmation of efficient bisulfite conversion, PCR sequencing of six CpG sites of the *CNS1* region localised 4.2 kb upstream from the transcriptional start site of *IFNG* and five CpG sites in the *IFNG* promoter was performed by next generation sequencing (NGS). Therefore, the *IFNG* and *CNS1* PCR products were sequenced, performed by Seq-IT GmbH & Co. KG in Kaiserslautern. Raw data were provided from forward and reverse reads. Data were evaluated through Geneious 7.1.9. The mean methylation was calculated from every single CpG site from all available reads. Per sample and region between 166503 and 237445 reads were used for calculation.

3.26 Statistical analysis

Data were examined for normal distribution and followed by an equivalent outlier test. For the comparison of two groups either a parametric or nonparametric t test was performed. For the calculation of statistical significance between groups displaying paired observations either the Wilcoxon matched pairs test or the paired t test was used depending on the distribution of the data. Three or more groups were compared by one-way analysis of variance (ANOVA) or a Kruskal-Wallis test. Linear regression analysis was performed to determine the correlation of two parameters.

P-values ≤0.05 were considered to be statistically significant. Statistical analyses were performed using GraphPad Prism 5.04 software (GraphPad Software, San Diego California USA).

4 Results

4.1 iNKT cells in HCV infection

Initially, iNKT cells have been studied intensively in mice utilising either antibodies against the T cell receptor chains Vα14 and Vβ8.2, Vß7 and Vß2 or αGalCer loaded CD1d dextramers. Since iNKT cells recognise αGalCer presented by the MHC class I like molecule CD1d, dextramers of αGalCer loaded CD1d can be used for the analysis of iNKT cells. Therefore, human PBMCs were stained for iNKT cells utilising a commercially available fluorescently labelled αGalCer loaded CD1d dextramer. By flow cytometric analyses iNKT cells were identified after exclusion of dead cells and doublets. CD1d dextramer$^+$ NKT cells were congruent with the described human invariant T cell receptor chains expressed by iNKT cells as they solely express Vα24 in combination with Vβ11 (figure 4.1). Further analysis of iNKT cells in this study were focused on invariant CD1d restricted cells that were detectable via a αGalCer loaded CD1d dextramer.

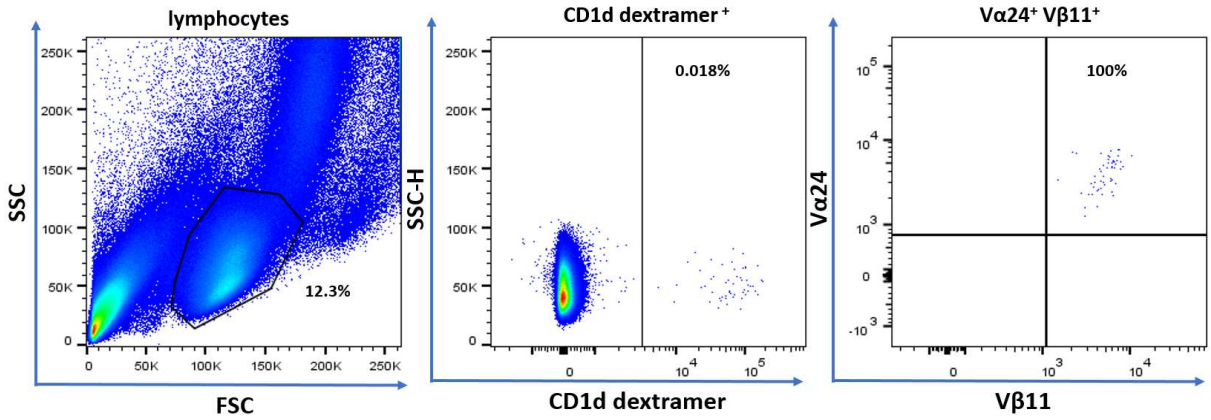

Figure 4.1: Identification of human iNKT cells

To determine iNKT cells using flow cytometry, lymphocytes were identified and dead cells, doublets as well as CD19$^+$ B cells were excluded. iNKT cells were identified as CD1d dextramer$^+$ cells and subsequently analysed for the expression of the T cell receptor chains Vα24 and Vβ11.

In contrast to iNKT cells, identification of non-invariant NKT cells, that are characterised by a diverse TCR repertoire, with existing methodology is challenging. Non-invariant NKT cells are commonly defined by their CD1d-restriction and the absence of the invariant TCR chains, as a unique surface marker to distinguish them from iNKT cells is still lacking (reviewed in Rhost et al. 2012). Therefore, the analysis of non-invariant NKT cell was not included in this study.

4.1.1 Frequency of iNKT cells in HCV-RNA positive and HCV-RNA negative PWID

The role of iNKT cells for the outcome of HCV infection is poorly defined and decreased iNKT cell frequencies have been reported in chronically HCV infected patients, however, contradicting reports exist. Therefore, iNKT cell frequencies in HCV infection were re-evaluated in a high-risk cohort of PWID. Since injection drug use represents the most important risk factor for HCV infection in industrialised countries as Germany, we utilised this cohort to comparatively study PWID developing chronic infection and individuals that spontaneously resolve HCV infection. In this study, a total of 61 individuals with a history of injection drug use were analysed including 28 HCV-RNA positive and 33 anti-HCV positive PWID with resolved HCV infection (HCV-RNA negative). Significantly reduced NKT cell frequencies have been observed early in HIV infection (Fernandez et al. 2014). Therefore, no HCV-HIV coinfected individuals were included to focus on HCV mediated effects. Patient characteristics of individuals included in the analysis of iNKT cells are summarised in table 4.1.

Table 4.1: Patient characteristics of PWID included in the iNKT cell study

complete cohort	HCV-RNA positive	HCV-RNA negative
n	28	33
mean age in years (range)	35 (22-55)	39 (22-53)
male (%)	23 (82.1%)	30 (90.1%)
HCV genotype 1 (%)	12(42.9%)	n.d.
HCV genotype 3 (%)	12(42.9%)	n.d.
other HCV genotypes or unknown (%)	4 (14.3%)	n.d.
median viral load in IU/mL (range)	582900 (615-34010000)	n.d.
anti-HIV positive (%)	0 (0%)	0 (0%)

n.d. not determined

iNKT cell frequencies in PWID ranged from undetectable to 0.23% and no significant difference in frequency could be observed between PWID with resolved HCV infection and HCV-RNA positive individuals. The median frequency was slightly higher in the HCV-RNA positive group with 0.032% compared to 0.017% in the HCV-RNA negative group (figure 4.2 A). Furthermore, a weak nonsignificant correlation between age and iNKT cell frequencies was observed in our cohort ($p=0.083$, $r^2=0.050$, figure 4.2 B). In addition, iNKT cell frequencies were not associated with the gender of patients, even though this analysis was limited due to

the small percentage of female PWID in this cohort. Moreover, no association of iNKT cell frequencies and viral load in HCV-RNA positive individuals could be determined (data not shown).

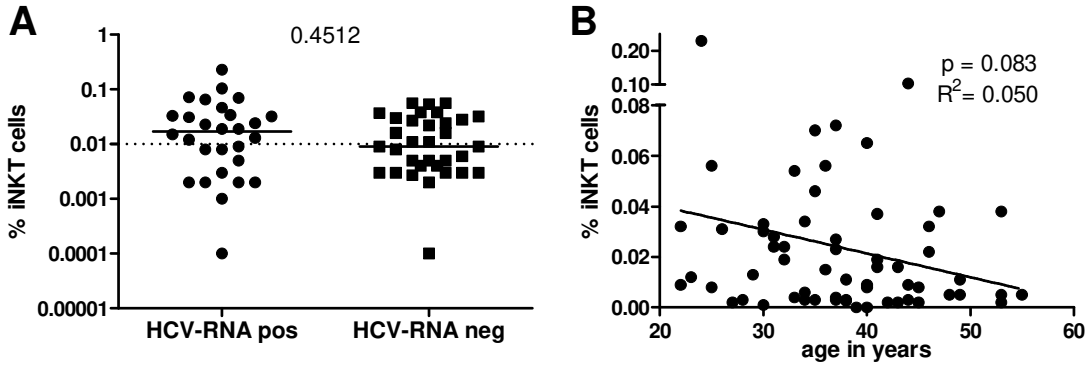

Figure 4.2: iNKT cell frequencies in HCV-RNA positive and HCV-RNA negative PWID

iNKT cell frequencies were determined via a CD1d dextramer staining of PBMCs from 28 HCV-RNA positive and 33 HCV-RNA negative individuals. Displayed is the percentage of iNKT cells from single, viable lymphocytes after the exclusion of $CD19^+$ B cells. To apply a logarithmic scale the limit of detection was defined as 0.0001% for individuals with no measurable iNKT cells frequencies. The dotted line at 0.01% represents the cut off for PWID that were included in further analysis. The p-value was calculated by nonparametric Mann Whitney test and the median is depicted (A). Moreover, the frequency of iNKT cell was correlated with the age of each analysed PWID independent of their HCV status. Linear regression was performed and the p-value as well as the R^2 is indicated (B).

Since iNKT cell were not measurable in all analysed individuals only individuals with previously detectable iNKT cells above the defined cut off of 0.01% were further included in phenotypical as well as functional analysis.

4.1.2 Phenotype of iNKT cells in HCV-RNA positive and HCV-RNA negative PWID

In HCV infection, various NK cell phenotypes have been repeatedly associated with HCV infection outcome. Moreover, differences in expression of differentiation or exhaustion markers on T cells have been linked to HCV infection outcome. Since elaborate phenotypical analyses of iNKT cells that share both characteristics of NK and T cells in HCV infection are lacking we aimed to comparatively analyse the phenotype of iNKT cells from HCV-RNA positive and HCV-RNA negative PWID. iNKT cells were defined as CD1d dextramer positive lymphocytes and were analysed for their expression of markers commonly associated with the NK cell linage like CD161, receptors of the CD94/NKG2 family and KIR2DL3 exemplifying the receptors of the KIR family. Furthermore, CD161 has been initially described as a NKT defining cell marker. In addition, to addressing the expression of the exhaustion marker PD1, that has been linked to T cell exhaustion in HCV infection, the expression of several

differentiation as well as activation markers such as CD57, CD127 and CD38 were analysed on iNKT cells (figure 4.4). By flow cytometric analysis lymphocytes were identified and subsequently dead cells as well as doublets were excluded. Following an exclusion of $CD19^+$ B cells due to unspecific staining of B cells by the dextramer, iNKT cells were defined. The gating strategy for iNKT cells and selected surface antigens CD38, PD1 and NKG2A, is shown in figure 4.3.

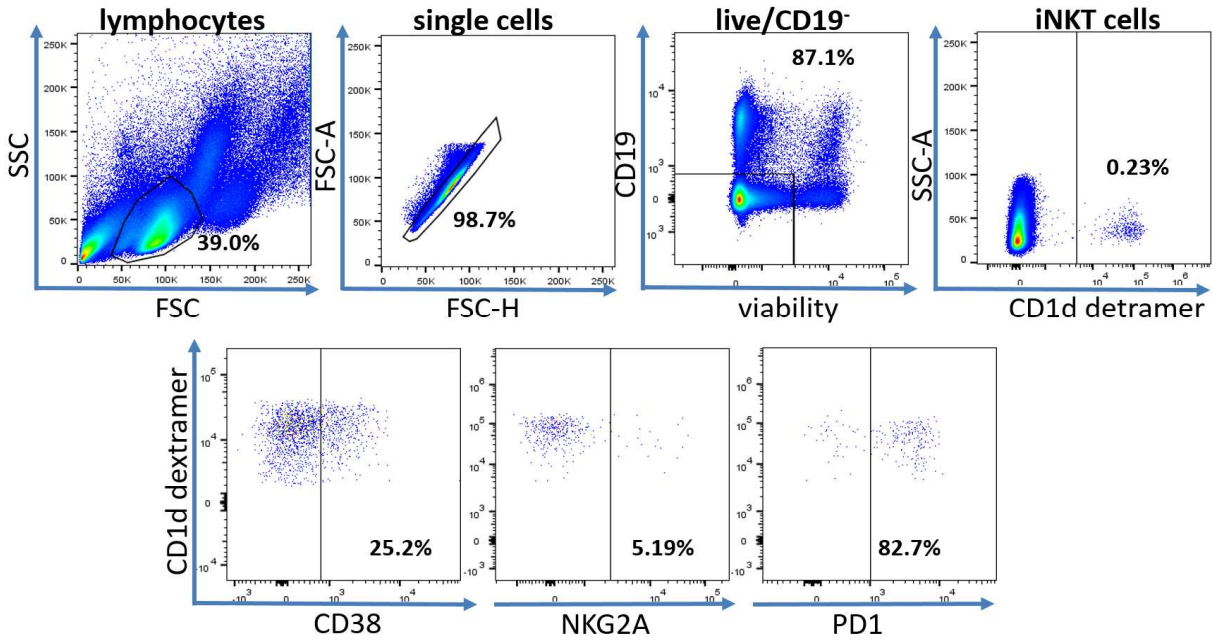

Figure 4.3: Representative gating strategy for iNKT cells in PWID

Representative gating strategy of the analysis of iNKT cells from chronically HCV infected PWID and PWID with spontaneously resolved HCV is depicted. Cryopreserved PBMC were thawed and iNKT cells were defined through CD1d dextramer staining as $CD19^-$, live, single lymphocytes. Subsequently the frequency of iNKT cells expressing NKG2A, NKG2D, KIR2DL3, CD38, CD127, CD57, PD1 and BTLA on iNKT cells were determined. An Exemplary gating for CD38, NKG2A and PD1 is shown.

As previously reported, high frequencies of $CD161^+$ cells in the iNKT cell population could be confirmed with a median frequency of 85% (Lee et al. 2002). Expression of the NK cell receptors NKG2A, NKG2D and KIR2DL3 on iNKT cells, previously described to be differentially regulated on NK cells in HCV infection, did not significantly differ between groups. Interestingly, out of the nine tested receptors the activation marker CD38 was significantly higher expressed on iNKT cells of HCV-RNA positive PWID (mean: 24.5%) compared to HCV-RNA negative PWID (mean: 8.9%; p=0.0008). Despite this activated phenotype in PWID with chronic HCV infection, iNKT cells expressed high levels of CD127 and predominantly lacked CD57, irrespective of the infection status. Moreover, no difference in expression of the exhaustion markers PD1 and BTLA could be observed between groups.

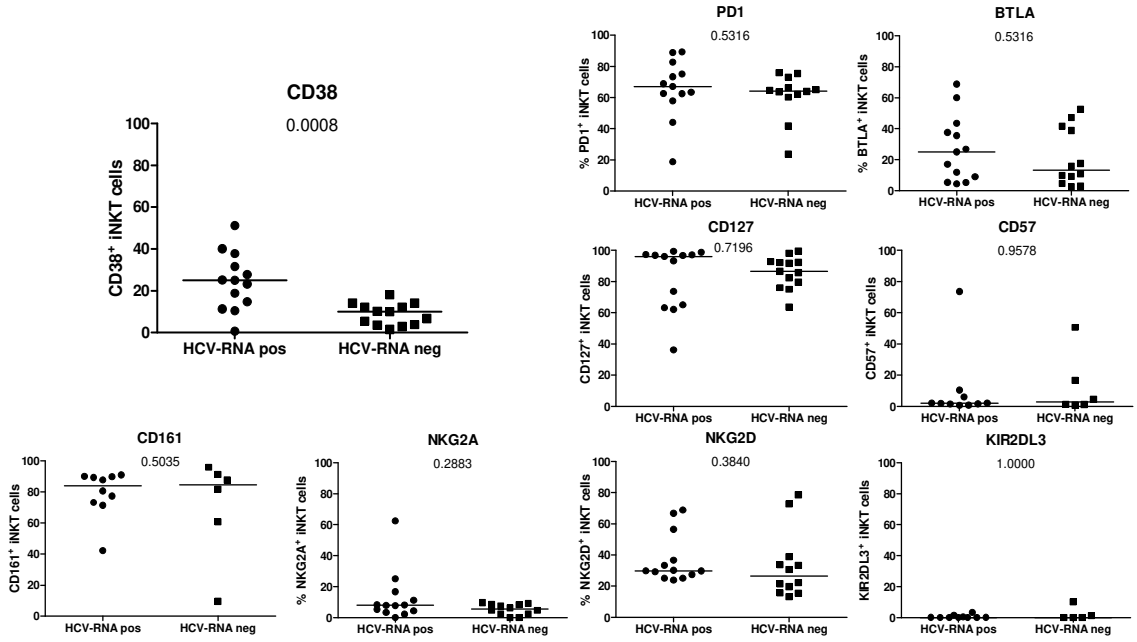

Figure 4.4: Phenotypical comparison of iNKT cells from HCV-RNA positive and HCV-RNA negative PWID

Expression of the NK cell markers CD161, NKG2A, NKG2D and KIR2DL3 (lower panel) as well as the differentiation markers CD127, CD57 (middle panel) and markers associated with exhaustion and activation like CD38, PD-1 and BTLA (top panel) were analysed by flow cytometry on iNKT cells. In total 13 HCV-RNA positive and 13 HCV-RNA negative PWID were analysed for their iNKT cell phenotype. Samples size between panels varies due to limitation in the availability of patient samples. If no or extremely low expression was observed as this was the case for CD57 and KIR2DL3 or if analysis was performed as proof of principle which holds true for CD161, data acquisition was discontinued, accounting for further differences in sample size. P-values were calculated by nonparametric Mann Whitney test and the median is depicted.

To determine if this activated phenotype is solely seen on iNKT cells, CD38 expression was analysed on CD1d dextramer negative lymphocytes. No difference in CD38 frequency could be observed between HCV-RNA positive and negative PWID on non iNKT lymphocytes (appendix figure 6.1). This indicates that solely iNKT cells in chronic HCV infection are in a pre-activated state.

Four different human iNKT cell subsets have been previously described in the periphery, which are defined by the expression of the cell surface antigens CD4 and CD8. To address a possible change in iNKT cell subset distribution during HCV infection $CD4^+/CD8^-$ ($CD4^+$), $CD4^-/CD8^+$ ($CD8^+$), double negative (DN) and double positive (DP) iNKT cells were determined in HCV-RNA positive and HCV-RNA negative PWID. However, the observed frequency of each subset was similar to previously described proportions in healthy individuals. Moreover, iNKT cell subset distribution did not significantly differ between HCV-RNA positive and HCV-RNA negative PWID (figure 4.5).

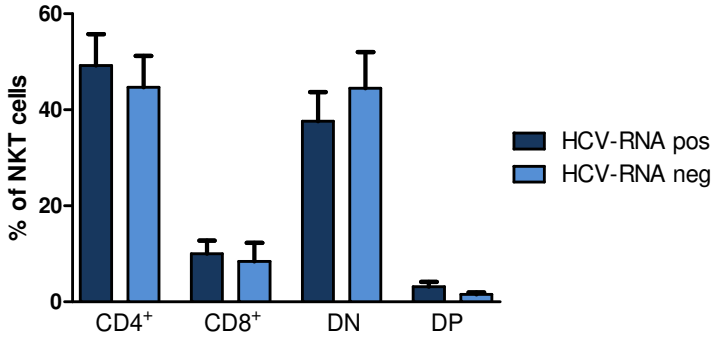

Figure 4.5: iNKT cell subset distribution in HCV-RNA positive and HCV-RNA negative PWID

iNKT cell subset distribution was analysed in 14 HCV-RNA positive and 10 HCV-RNA negative PWID. Frequency of $CD4^+/CD8^-$ ($CD4^+$), $CD4^-/CD8^+$ ($CD8^+$), double negative (DN) and double positive (DP) iNKT cells was determined. Statistical analysis revealed no associations and the mean ± standard error of mean (SEM) is depicted.

4.1.3 Functional analysis of iNKT cells in HCV-RNA positive and HCV-RNA negative PWID

Since iNKT cells of chronically HCV infected individuals displayed an activated phenotype, further analysis is needed to investigate if this translates into different functional properties of iNKT cells from HCV-RNA positive and HCV-RNA negative PWID. The proliferation potential of iNKT cells was determined by treatment of PBMCs with αGalCer, an exogenous ligand for iNKT cells. Robust iNKT cell expansion was induced after 10 days of culture in both groups. Independent of HCV infection status, iNKT cell frequencies were significantly increased after *in vitro* stimulation compared to iNKT cell frequencies *ex vivo* (****$p \leq 0.0001$, figure 4.6 A). The expansion potential of iNKT cells was calculated as fold change of iNKT cell frequencies determined *ex vivo* and after *in vitro* expansion. A slight tendency towards an increased expansion capacity of iNKT cells from HCV-RNA positive individuals could be observed in comparison to HCV-RNA negative PWID ($p=0.1662$, figure 4.6 B). Expansion of iNKT cells in both HCV-RNA negative PWID and PWID spontaneously resolving HCV infection was significantly associated with upregulation of the activation marker CD38 and downregulation of CD127 (figure 4.6 D+E). Downregulation of CD127 has been associated with T cell activation in HIV infection (Benito et al. 2008). Therefore, the determined iNKT cell phenotype of $CD38^{high}$ $CD127^{low}$ indicates that iNKT cells become highly activated during expansion with αGalCer irrespective of infection outcome.

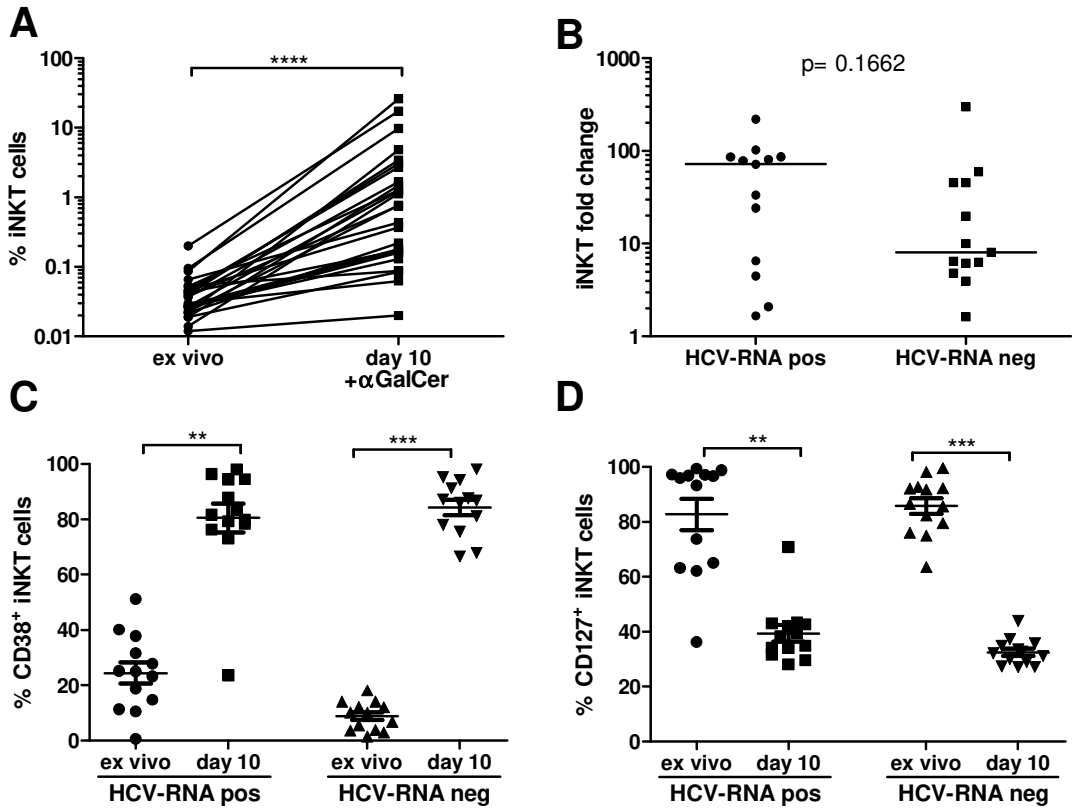

Figure 4.6: Proliferation potential of iNKT cells from HCV-RNA positive and HCV-RNA negative PWID

PBMCs from 13 HCV-RNA positive and 13 HCV-RNA negative PWID were analysed for their proliferation potential. Frequencies of iNKT cells were determined *ex vivo* and after 10 days of *in vitro* expansion with IL2 and αGalCer (A). Fold change of CD1d dextramer$^+$ iNKT cells between day 0 and day 10 was calculated (B). Furthermore, the frequency of CD38$^+$ iNKT cells and CD127$^+$ iNKT cells was determined after expansion (C+D). For statistical analysis Wilcoxon's matched-pairs signed rank test was used (****p≤0.0001; A). For comparison of differences in iNKT fold change the p-value was calculated by nonparametric Mann Whitney test (B). One-way ANOVA was used for the statistical comparison of four groups (C and D **p≤0.01, ***p≤0.001).

As the distinct activated phenotype observed in chronic HCV infection had only marginal impact on the proliferation capacity of iNKT cells, functional characteristics of iNKT cells of patients with chronic and resolved HCV infection were further addressed. iNKT cells are characterised by their ability to rapidly produce cytokines such as IFNγ or IL2 and the release of cytotoxic granules upon activation. Expression of CD107a, a marker for degranulation and cytotoxicity, as well as the production of IFNγ and IL2 of iNKT cells was analysed in 14 HCV-RNA positive PWID and 13 HCV-RNA negative PWID with resolved HCV infection in response to stimulation with PMA and Ionomycin. The function of iNKT cells was examined either *ex vivo* or after 10 days of *in vitro* expansion (figure 4.7). *Ex vivo* functional analysis revealed a high IFNγ production potential of iNKT cells while the degranulation ability was low and only detectable in approximately half of the analysed patient samples. However,

independent of HCV status, iNKT cells produced similar levels of IFNγ, IL2 and CD107a. Interestingly, the potential of iNKT cells to produce IL2 as well as their degranulation ability determined by CD107a expression was significantly improved by stimulation of PBMCs with αGalCer over 10 days. Even though no significant difference in iNKT functionality regarding production of IFNγ, IL2 and expression of CD107 could be observed, there was a slight tendency towards increased IFNγ production in HCV-RNA positive individuals after *in vitro* expansion.

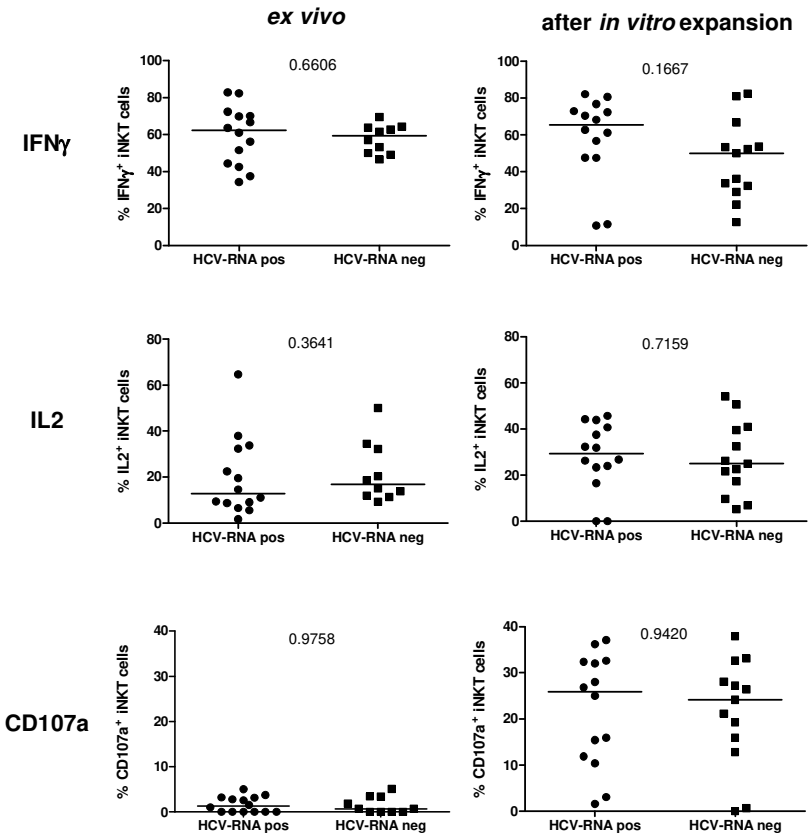

Figure 4.7: Cytokine profile of iNKT cells from HCV-RNA positive and HCV-RNA negative PWID

The frequency of IFNγ+, IL2+ and CD107a+ iNKT cells in 14 PWID with chronic or 13 with resolved HCV infection was measured via intracellular cytokine staining and flow cytometric analysis. Samples were either stimulated with PMA (10 ng/mL)/Ionomycin (1 µg/mL) for 5 hours in the presence of BFA (100 ng/mL) *ex vivo* (C) or their functionality was addressed after 10 days of expansion with αGalCer. Samples with less than 10 detectable iNKT cells were excluded from the *ex vivo* analysis. P-values were calculated using a nonparametric Mann Whitney test and the median is depicted.

4.1.4 Combination of interleukins induces CD38 expression on iNKT cells *in vitro*

ISGs induced by type I or type III IFNs as IFNα or IFNλ3 are upregulated in HCV infected liver and increased endogenous production of IFNλ by HCV infected cells has been reported (Marukian et al. 2011, Park et al. 2012). Although IFNs mediate antiviral effects, chronic HCV infection has been associated with increased IFNα

levels. Continuous induction of ISGs seen in chronic infection lead to decreased responsiveness to PEGylated IFNα based therapies (Sarasin-Filipowicz et al. 2008). Therefore, it was addressed if IFNα or IFNλ have a direct influence on iNKT cells. Moreover, potential effects of a combination of IL12, IL15 and IL18, previously reported to activate NK cells, were assessed. PBMCs from healthy individuals were stimulated over a period of 24 hours and the frequency of CD38 as a marker for activation was determined on iNKT cells by flow cytometry. While no difference in activation of iNKT cells could be observed in response to IFNα or IFNλ3 stimulation (figure 4.8 A+B), treatment with a combination of IL12, -15 and -18 led to a significant increase in CD38$^+$ iNKT cells (p=0.0030, figure 4.8 C). Moreover, an additive effect of IL12 and IL15 could be observed (figure 4.8 D). In contrast to NK cells, IL18 had no effect on iNKT cell activation and the observed increase was solely mediated by IL12 and IL15. However, this IL12, IL15 mediated activation was not unexpected since NKT cells harbour various NK cell properties.

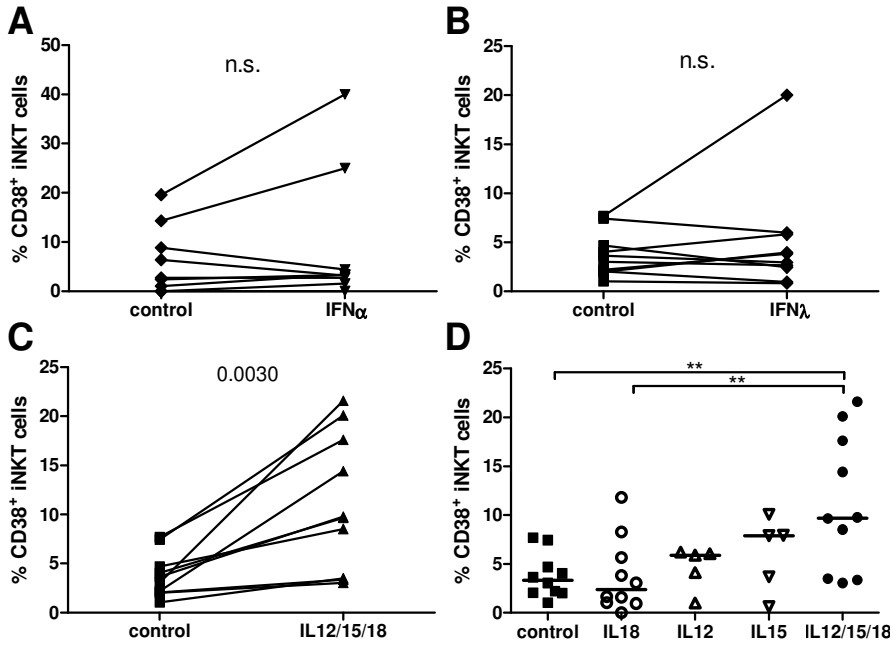

Figure 4.8: Influence of IFNα, IFNλ3 and IL12, -15 and -18 on iNKT cell activation

PBMCs from healthy individuals were stimulated over 24 hours with or without 100 IU IFNα/mL (A) and the expression of CD38 on iNKT cells was determined by flow cytometry. Furthermore, PBMCs were stimulated for 24 hours in media supplemented with IFNλ3 (1 000 IU/mL) (B), IL12 (10ng/mL), IL15 (100 ng/mL) or IL18 (100 ng/mL) alone or in combination (10 ng/mL, 100 ng/mL, 50 ng/mL) (C+D). Activation of iNKT cells was determined by the measurement of the frequency of CD38$^+$ iNKT cells. Significance was calculated by paired t-test and comparison of more than two groups was done using One-way ANOVA **p≤0.01.

4.1.5 Expression of the iNKT cell ligand CD1d is independent of HCV infection outcome

In HIV infection CD1d is downregulated by the Nef protein and this downregulation is associated with reduced iNKT cell activation (Chen et al. 2006). Since we observed an increase in iNKT cell activation displayed by CD38 expression we hypothesised that in chronically HCV infected patients CD1d might be upregulated in comparison to patients with resolved infection. To identify cells, capable of presenting glycolipids to iNKT cells in the periphery, PBMCs from five healthy individuals were analysed by flow cytometry for their cell surface expression of CD1d. In line with previous reports, suggesting that CD1d is most prominently expressed on antigen presenting cells (Roark et al. 1998), monocytes showed the highest CD1d expression of all analysed cells in the periphery. Moreover, CD1d expression could be detected in B cells while T and NK cells lacked CD1d on the cell surface (figure 4.9 A). Since B cells and monocytes displayed detectable CD1d on their surface, both cell types were subsequently studied in HCV infected individuals. For that purpose, CD1d expressing monocytes and B cells from 13 PWID with chronic HCV infection and 13 PWID with resolved infection were identified by flow cytometry. However, the analysis between HCV-RNA positive and HCV-RNA negative individuals revealed no significant differences in the expression of CD1d neither on monocytes nor on B cells (figure 4.9 B+C).

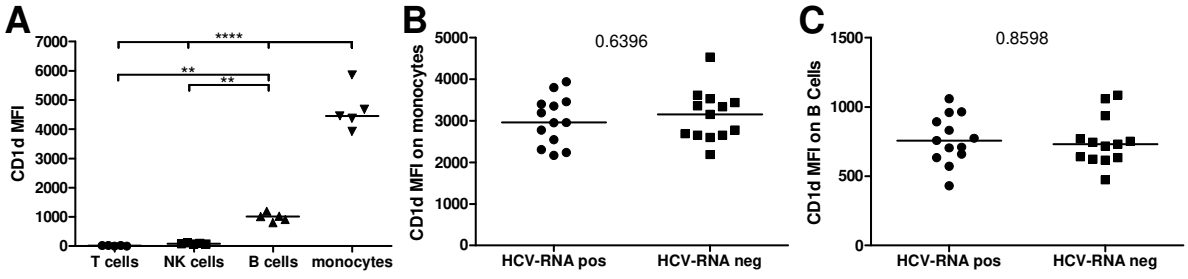

Figure 4.9: Expression of CD1d on peripheral cells

CD1d expression on $CD3^+$ T cells, $CD56^+$ NK cells, $CD19^+$ B cells and $CD14^+$ monocytes from five healthy individuals was determined. As monocytes have a different cell morphology they were first identified through the FSC/SSC scatter plot as a population distinct from lymphocytes because of their increased size. Afterwards populations were defined by their expression of cell surface markers specific to the cell type (A). One-way ANOVA $^{**}p \leq 0.01$ $^{****}p \leq 0.0001$ was used to analyse statistical significance. Since monocytes displayed the highest CD1d mean fluorescence intensity (MFI) in the periphery, PBMCs from 13 chronically HCV infected PWID and 13 PWID with spontaneously resolved HCV infection were analysed for CD1d MFI (B). Moreover, CD1d MFI on B cells was examined between groups. Unpaired t-test was performed to determine significance and the median is depicted.

4.1.6 IFNα mediates upregulation of the iNKT cell ligand CD1d *in vitro*

While no direct effect of IFNα and IFNλ stimulation on iNKT cell activation could be detected, IFNs might mediate other effects and thus indirectly contribute to iNKT cell activation. Classical MHC class I expression in the hepatic compartment is low compared to other tissues, whereas hepatocytes express high levels of CD1d (Agrati et al. 2005). Since IFNs are upregulated in HCV infected liver we hypothesised that they might contribute to differences in CD1d expression. Therefore, the influence of IFNα, IFNλ or the combination of IL12, -15 and -18 on CD1d expression was determined. Expression analysis was focused on monocytes as they displayed the highest CD1d expression in the periphery. No influence on CD1d expression could be detected after 24 hours of stimulation with IFNλ or the combination of IL12, -15 and -18 (figure 4.10 C+D). Interestingly, it could be demonstrated that CD1d is significantly increased on monocytes upon IFNα treatment (p=0.0120 figure 4.10 B), suggesting that high IFNα levels in the liver might mediate upregulation of CD1d on hepatocytes and subsequently lead to the activation of iNKT cells in the liver.

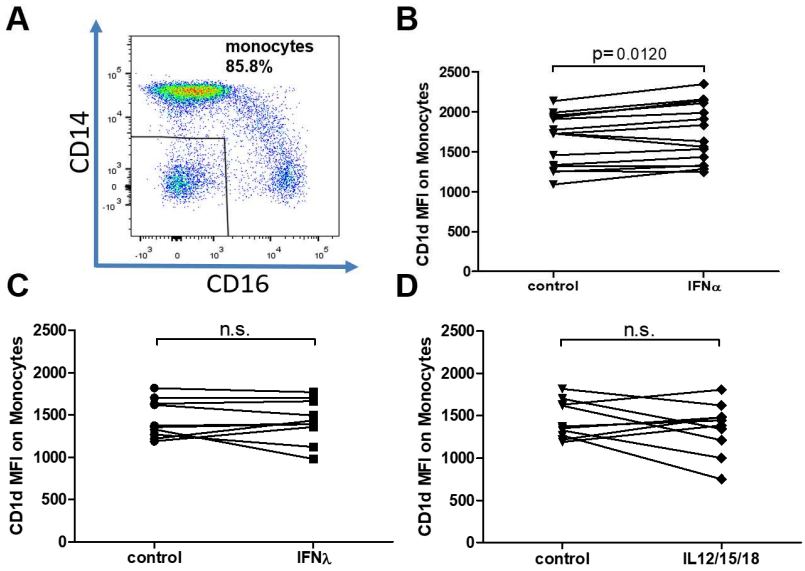

Figure 4.10: Influence of IFNα, IFNλ3 and IL12, -15 and - 18 on CD1d expressing monocytes

PBMCs from healthy individuals were stimulated for 24 hours in media supplemented with either 100 IU IFNα/mL or IFNλ3 (1000 IU/mL) or IL12/15/18 (10 ng/mL, 100 ng/mL, 50 ng/mL). The influence of the treatment on the median fluorescence intensity of CD1d on monocytes was determined. Significance was calculated by paired t-test.

4.1.7 Monocytes influence the expansion and activation of iNKT cells

As monocytes were the most prominent cell type in the periphery expressing CD1d their impact on iNKT cell expansion and activation was addressed. We hypothesised that absence of CD1d expressing monocytes might lead to reduced expansion of

iNKT cells since cells able to present CD1d restricted glycolipid ligands to iNKT cells are decreased. Thus, PBMCs from 15 healthy individuals were stimulated with αGalCer either in the presence or absence of monocytes, followed by flow cytometric analysis. iNKT cell frequencies as well as CD38 expression as a marker for activated iNKT cell was determined. Indeed, a strong tendency towards a lower iNKT cell expansion capacity was observed in the monocyte depleted culture (p=0.0730, figure 4.11 A). Furthermore, the analysis indicates that a depletion of monocytes has a major impact on the activation of iNKT cells since significantly reduced frequencies of $CD38^+$ iNKT cells (p=0.0038) as well as a reduction in CD38 surface density (p=0.0203) on iNKT cells were detected (figure 4.11 B+C). These results proof that CD1d expressing cells are needed for proper iNKT cell expansion as well as iNKT cell activation.

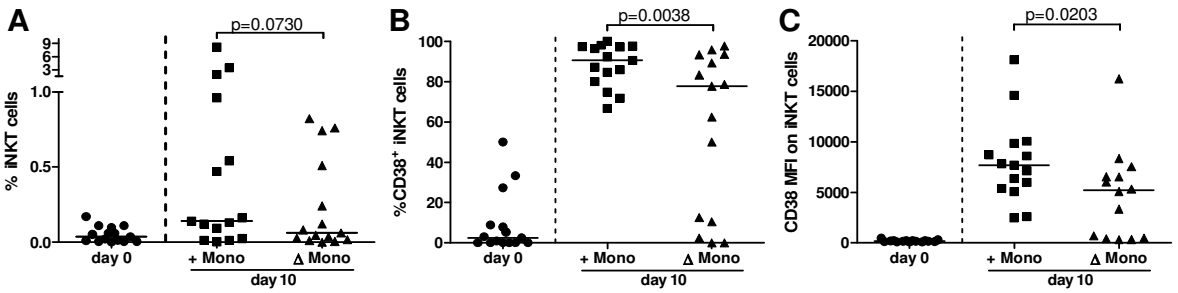

Figure 4.11: Influence of monocytes on the expansion and activation of iNKT cells

PBMCs from 15 healthy individuals were expanded over a period of 10 days with IL2 (25 IU/mL) and αGalCer (1 µg/mL) in the presence or absence of monocytes. Monocytes were depleted by adherence to plastic. iNKT cell frequencies (A) and frequency of CD38 expressing iNKT cells as well as CD38 MFI on iNKT cells (B+C) was determined directly after thawing and after 10 days of culture. The median is shown and statistical significance was determined by Wilcoxon signed rank test *p≤ 0.05, **p≤ 0.01.

4.1.8 CD1d is upregulated in HCV infected liver

Previous reports demonstrated high NKT cell frequencies in the liver of mice (Eberl et al. 1999). Therefore, we tested if iNKT cell frequencies are similarly elevated in the human system. iNKT frequencies were determined in liver infiltrating lymphocytes and compared to the above stated iNKT cell frequencies in the periphery. In contrast to studies in mice, an enrichment of iNKT cells in the human liver could not be observed (figure 4.12 A+B). Moreover, studies in mice showed a proinflammatory role of CD1d restricted NKT cells contributing to hepatic inflammation and liver fibrosis (Ishikawa et al. 2011). Since HCV is a hepatotropic infection, upregulation of CD1d in the liver could contribute to iNKT cell activation. Therefore, the expression of CD1d was analysed in the liver. In cooperation with the clinic for Gastroenterology

and Hepatology at the University Hospital Essen an expression profile of CD1d from liver biopsies was generated to compare messenger RNA (mRNA) levels between 54 HCV infected patients and 23 patients with HBV infection. The mRNA expression of CD1d was significantly higher in HCV infected individuals compared to HBV infected patients ($p \leq 0.05$, figure 4.12 C). As HCV infection in contrast to HBV infection is known to induce a strong type I IFN response in the liver, our observed effect of IFNα mediated upregulation of CD1d on monocytes in the periphery could therefore possibly account for the difference in CD1d mRNA level between HCV and HBV infected patients.

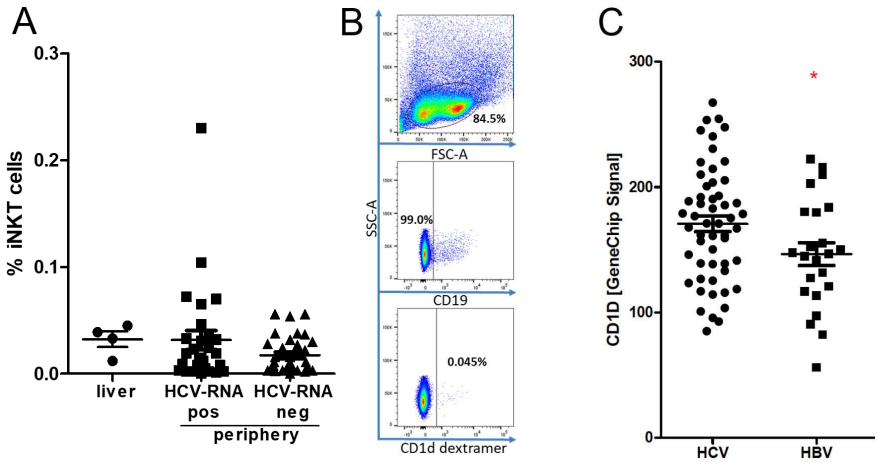

Figure 4.12: iNKT frequencies and CD1d mRNA levels in the liver

iNKT cell frequencies were analysed in four liver samples as CD1d dextramer^{+} CD19^{-} lymphocytes (B). Frequencies in the liver were compared with initial determined iNKT cell frequencies in the periphery of HCV-RNA positive and HCV-RNA negative PWID (A). One-way ANOVA was used to analyse statistical significance. Furthermore, CD1d mRNA levels were compared between liver biopsies of 54 HCV and 23 HBV infected patients. Data of CD1d mRNA levels were kindly provided by the Clinic for Gastroenterology and Hepatology at the University Hospital Essen. The mean ± SEM is depicted.

4.2 NK cell in HCV infection

While iNKT cells are still poorly covered by scientific reports in HCV infection there is growing evidence for the importance of NK cells for HCV infection outcome. Different genetic association studies revealed that genetically determined combinations of NK cell-receptors and their ligands are associated with differential outcome of HCV infection. NK cell function is regulated by a set of different inhibitory and activating receptors including killer-cell immunoglobulin-like receptors (KIRs). Khakoo et al. (2004) demonstrated that PWID homozygous for KIR2DL3 and its ligand HLA-C1 are significantly enriched in patients who spontaneously resolve HCV infection. Therefore, the aim was to analyse whether certain KIR/KIR-ligand combinations are associated with HCV infection outcome in our PWID cohort and if identified KIR/KIR-ligand combinations correlate with functional properties of NK cells.

4.2.1 PWID cohort

As previously described injection drug use is the most common risk factor for HCV infection in high income countries (Nelson et al. 2011). Consistent with these findings, we observed that with approximately 81% the majority of our PWID cohort (n=266) were HCV antibody positive (table 4.2). Of the analysed PWID, 57% had detectable HCV RNA consistent with ongoing viral infection. In turn, 24% were HCV-RNA negative with detectable HCV antibody titres, consistent with spontaneous immune control of HCV infection. Interestingly, the remaining 19% were HCV seronegative despite continuous high-risk behaviour. The high frequency of sharing injection materials among PWID suggests that at least parts of this group were also exposed to HCV infection. Accordingly, these exposed anti-HCV seronegative PWID were resistant to infection or achieved immune control during an early phase prior to seroconversion. The ratio between male and female patients included in this study did not significantly differ between the three subgroups. However, anti-HCV negative PWID were significantly younger than HCV-RNA positive patients (appendix figure 6.2, $p \leq 0.05$).

Table 4.2: Patient characteristics of PWID included in the KIR study

	Germany			North America	
complete cohort	**HCV-RNA positive**	**HCV-RNA negative**	**anti-HCV negative**	**HCV-RNA positive**	**HCV-RNA negative**
n	151	64	51	267	75
mean age in years (range)	38 (18-61)	38 (20-53)	34 (18-49)	33 (18-73)	35 (19-59)
male (%)	116 (76.8%)	56 (87.5%)	38 (74.5%)	168 (62.9%)	28 (37.3%)
HCV genotype 1 (%)	84 (55.6%)	n.d.	n.d.	158 (59.2%)	n.d.
HCV genotype 3 (%)	64 (42.4%)	n.d.	n.d.	38 (14.2%)	n.d.
other HCV genotypes or unknown (%)	3 (2%)	n.d.	n.d.	71(26.6%)	n.d.
median viral load in IU/mL (range)	852848 (621-7778000)	n.d.	n.d.	862456 (200-40500000)	n.d.
anti-HIV positive (%)	5 (3.3%)	1 (1.5%)		31 (11.6%)	12 (16%)

GT: genotype, IU: international units, n.d.: not determined

4.2.2 KIR/KIR-ligand combinations associated with HCV infection outcome in a high-risk group of PWID

A previous study in our group had the aim to identify if certain KIR/KIR-ligand constellations play a role in HCV infection outcome in a high-risk cohort of PWID. Therefore, 266 treatment naïve well characterised patients comprising 151 HCV-RNA positive PWID, 64 HCV-RNA negative PWID and 51 anti-HCV negative PWID were included in the genetic analysis (table 4.2). PWID were KIR and HLA class I typed at a 4-digit resolution level to allow discrimination between HLA-A and -B alleles encoding a Bw6, Bw4 80(I) or Bw4 80(T) motif. While all three motifs are seen on HLA-B alleles, HLA-A alleles only encode for Bw6 and Bw4 80(I).

An univariate analysis testing all known KIR/KIR-ligand constellations for possible associations with infection outcome of HCV, revealed that the KIR/KIR-ligand constellation of KIR2DL1/HLA-C2 (p=0.014) as well as KIR3DL1/HLA-Bw4 80(T) (p=0.003) was significantly enriched in seropositive HCV-RNA negative PWID compared to HCV-RNA positive PWID (table 4.3). In contrast to Khakoo et al. (2004), no association between the constellation KIR2DL3/HLA-C1 and HCV infection outcome was observed.

Table 4.3: Genetic association between KIR/KIR-ligands and HCV infection status in PWID

				univariate analysis		multivariate analysis		
gene	HCV-RNA positive	HCV-RNA negative	anti-HCV negative	p-value[a]	p-value[b]	p-value[a]	OR	95% CI
KIR2DL1/HLA-C2	9.9%	20.3%	9.8%	0.014	1.000	n.s.	-	-
KIR3DL1/HLA-Bw4 80(T)	25.8%	46.9%	29.4%	0.003	0.714	0.007	2.90	1.339 - 6.267

n.s. not significant, [a] HCV-RNA pos vs. HCV-RNA neg, [b] HCV-RNA pos vs. anti-HCV neg
OR: odds ratio; CI: confidence interval

Through a multivariate logistic regression analysis, the association between the genetic constellation of KIR3DL1/HLA-Bw4 80(T) and spontaneous resolution of HCV was confirmed (p=0.007). In contrast, the association of KIR2DL1/HLA-C2 with infection outcome was not verified by multivariate logistic regression analysis. Table 4.3 depicts all significant associations between KIR/KIR-ligand constellation and HCV infection status in a univariate and multivariate logistic regression analysis that were previously described in our group. Since no previous reports linking KIR3DL1/HLA-Bw4 80(T) to HCV infection outcome exist, a second genetic association study was performed in a separate North American PWID cohort. 342 PWID including 267 HCV-RNA positive and 75 HCV-RNA negative individuals were analysed for associations between KIR/KIR-ligand constellation and infection outcome (table 4.2). The genetic data of the North American cohort were kindly provided by Georg M Lauer. As seen in the German cohort the genetic combination of KIR3DL1/HLA-Bw4 80(T) was significantly enriched in HCV-RNA negative individuals compared to HCV-RNA positive PWID (44.0% vs. 28.8%; p=0.017) and thereby verified an association between KIR3DL1/HLA-Bw4 80(T) and spontaneous clearance of hepatitis C. Figure 4.13 illustrates that PWID encoding the combination of KIR3DL1 and HLA-Bw4 80(T) were enriched in PWID that spontaneously control HCV infection.

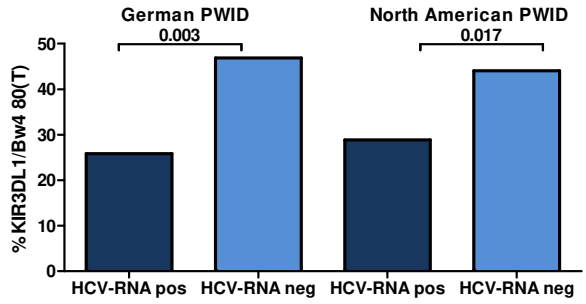

Figure 4.13: Frequency of the genetic combination of KIR3DL1/HLA-Bw4 80(T) in two PWID cohorts from Germany and North America grouped by infection outcome

The frequency of individuals with the KIR3DL1/Bw4 80(T) genotype in HCV-RNA positive PWID (dark blue) and HCV-RNA negative PWID (light blue) is shown in percent. P-values were calculated using Fisher's exact test.

In summary, our group could identify that the genetic combination of KIR3DL1/HLA-Bw4 80(T) was associated with spontaneous immune control of HCV infection in two separate cohorts.

4.2.3 Bw4 copy number is associated with HCV infection outcome

Since copy number of KIR-ligands were described to influence NK cell functionality (Kim et al. 2008) the PWID cohort described in table 4.2 was examined for possible association between HLA-Bw4 copy number and HCV infection status. PWID were grouped according to the HLA-Bw4 copy number into HCV-RNA positive PWID, HCV-RNA negative PWID and anti-HCV seronegative PWID either lacking a HLA-Bw4 allele, carrying one HLA-Bw4 allele or carrying two or more HLA-Bw4 alleles (table 4.4).

Table 4.4: Association between HLA-Bw4 copy number and HCV infection status in PWID

	HCV-RNA positive	HCV-RNA negative	anti-HCV negative	p-value[a]	p-value[b]
n	151 (100%)	64 (100%)	51 (100%)	n.s	n.s
no KIR3DL1	7 (4.6%)	4 (6.3%)	1 (2%)	n.s	n.s
KIR3DL1 + 0 Bw4	45 (29.8%)	14 (21.9%)	13 (25.5%)	n.s	n.s
KIR3DL1 + 1 Bw4	76 (50.3%)	27 (42.2%)	17 (33.3%)	n.s	n.s
KIR3DL1 + ≥ 2 Bw4	**23 (15.2%)**	**19 (29.7%)**	**20 (39.2%)**	**0.0299**	**0.0006**

n.s. not significant, a HCV-RNA pos vs. HCV-RNA neg, b HCV-RNA pos vs. anti-HCV neg

PWID expressing multiple Bw4 copies were significantly enriched in HCV-RNA negative PWID (29.7%) compared to HCV-RNA positive PWID (15.2%, p=0.0299). This difference was even more pronounced when comparing anti-HCV negative PWID (39.2%; p=0.0006) to HCV-RNA positive PWID. Figure 4.14 visualises the

genetic association between multiple Bw4 copy numbers and infection outcome. Frequency of patients harbouring two or more Bw4 copies in HCV-RNA positive, HCV-RNA negative and anti-HCV negative PWID is displayed.

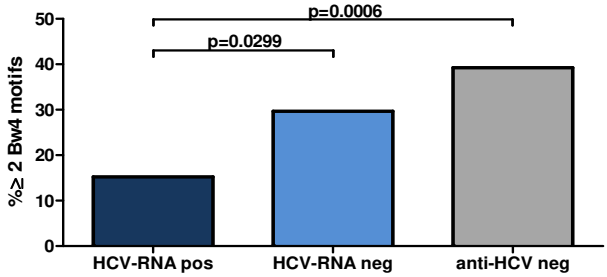

Figure 4.14: Frequency of PWID having ≥ 2 Bw4 motifs grouped by infection outcome
The frequency of individuals having two or more Bw4 motifs in HCV-RNA positive PWID (dark blue), HCV-RNA negative PWID (light blue) and anti-HCV seronegatives (grey) are shown in percent. P-values were calculated by Fisher's exact test.

4.2.4 Frequency and function of KIR3DL1$^+$ NK cells are independent of HCV infection status

Since an association between the genetic constellation of KIR3DL1/HLA-Bw4 80(T) as well as Bw4 copy number and HCV infection outcome was observed by Christine Thöns, the influence of KIR3DL1$^+$ NK cell was addressed further. Therefore, the aim of this project was to determine the functional consequences of this KIR3DL1/Bw4 80(T) genotype on the NK cells level in HCV infection. Moreover, the influence of the ligand for KIR3DL1, Bw4 was further investigated. For this reason, PBMCs from a representative subgroup of our PWID cohort was analysed through flow cytometry, including 30 HCV-RNA positive, 30 HCV-RNA negative and 30 anti-HCV negative PWID. PWID were preselected by their expression of the KIR3DL1 gene. For phenotypic analysis, KIR3DL1 expressing NK cells were defined through CD16 and CD56 expression of live, single lymphocytes that lack the surface markers CD3, CD14 and CD19. Effector functions such as IFNγ and TNFα production as well as CD107a expression of KIR3DL1$^+$ NK cells were determined. Therefore, PBMCs were stimulated with the HLA devoid target cell line K562 leading to the activation of NK cells through missing self-recognition. A representative gating strategy for NK cells and exemplary FACS plots from three individuals with different surface density of KIR3DL1 is shown in figure 4.15.

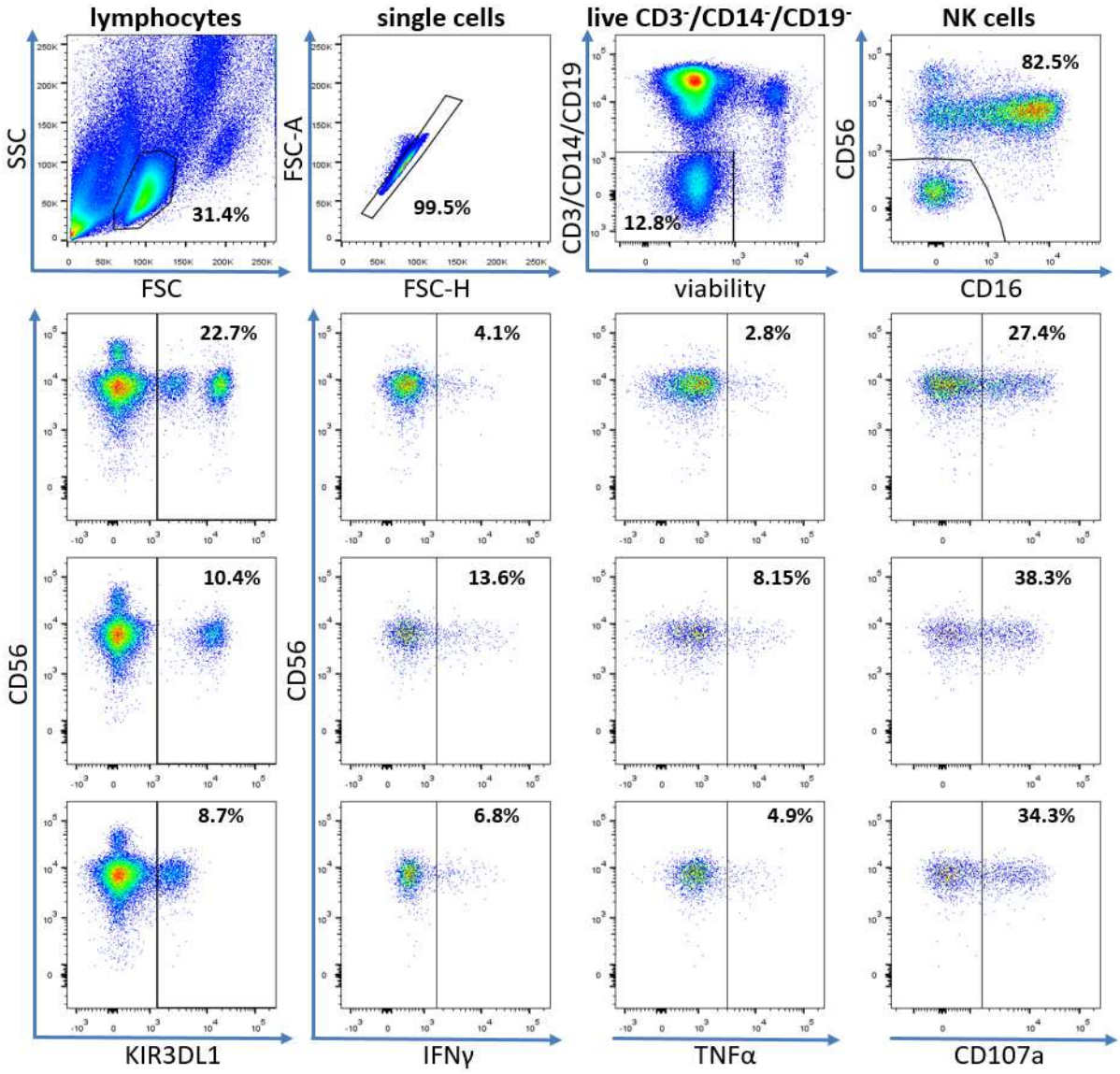

Figure 4.15 Representative gating strategy of the analysis of NK cell phenotype and function in PWID

A representative gating strategy of the analysis of NK cell phenotype and function from 30 HCV-RNA positive, 30 HCV-RNA negative and 30 anti-HCV seronegative PWID. Cryopreserved PBMCs were thawed and NK cells were defined through CD16 and CD56 expression of $CD3^-/CD14^-/CD19^-$, live, single lymphocytes. NK cells were further analysed for KIR3DL1 expression and function. IFNγ, TNFα and CD107a production was measured by intracellular cytokine staining after stimulation of PBMCs with K562 (10:1) for 5h. Flow cytometry results are shown from three different samples showing different KIR3DL1 expression on the cell surface of NK cells.

Comparison of KIR3DL1 expression on NK cells between HCV-RNA positive, HCV-RNA negative and anti-HCV negative PWID revealed no differences. Neither frequency of KIR3DL1 (figure 4.16 A) nor density defined by KIR3DL1 MFI (data not shown) on NK cells were associated with infection outcome. Furthermore, no functional differences could be observed between groups regarding IFNγ (figure 4.16 B) and TNFα production as well as CD107a expression of $KIR3DL1^+$ NK cells.

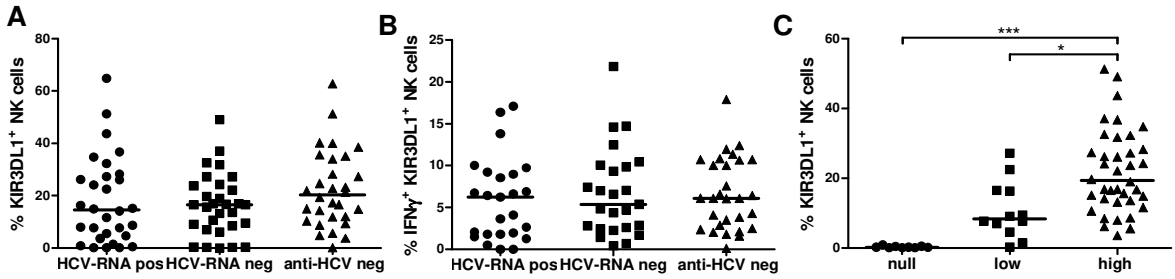

Figure 4.16: Phenotypic and functional analysis of NK cells from HCV-RNA positive, HCV-RNA negative and anti-HCV seronegative PWID

(A) The frequencies of KIR3DL1 expressing NK cells in HCV-RNA positive, HCV-RNA negative and anti-HCV negative PWID are depicted for all 90 analysed individuals. (B) IFNγ producing KIR3DL1$^+$ NK cells upon stimulation with K562 at an effector target ratio of 10:1 for 5h are shown. (C) The frequency of KIR3DL1$^+$ NK cells is depicted for HCV-RNA positive and HCV-RNA negative PWID according to the KIR3DL1 subtype expression level (null, low, high) as determined by a multiplex PCR assay (4). P-values were calculated by Kruskal Wallis test (*p≤ 0.05 and ***p≤0.001) and the median is depicted in every graph.

The KIR3DL1 gene locus is highly polymorphic with 79 described alleles to date. These alleles have been associated with different expression levels on the cell surface. While some alleles are linked to low or high density of KIR3DL1 expression on the cell surface, KIR3DL1*004 is not detectable on the cell surface due to intracellular retention. Therefore, a previous described multiplex PCR assay (Boudreau et al. 2014) with minor modifications was performed in cooperation with the Institute for Transplantation Diagnostics and Cell Therapeutics at the University Hospital Düsseldorf to characterise the KIR3DL1 gene in more detail. KIR3DL1 alleles were determined in 60 PWID that were included in the flow cytometry analysis. According to the provided PCR results all samples were divided into three groups. PWID encoding solely non-expressed KIR3DL1 alleles were grouped as KIR3DL1 null, while PWID encoding one low-expressed KIR3DL1 gene and absence of a high-expressed allele were grouped as KIR3DL1 low. The KIR3DL1 low group therefore contained PWID homozygous for low-expressed alleles as well as PWID with low-expressed alleles combined with the activating receptor KIR3DS1 or non-expressed alleles. The third group consists of patients that harboured at least one high expressed allele and was termed KIR3DL1 high. The KIR3DL1 allele subgroups defined by PCR were significantly associated with KIR3DL1 expression on the cell surface (figure 4.16 C). Frequency of KIR3DL1$^+$ NK cells as well as KIR3DL1 surface density (data not shown) was significantly higher in the KIR3DL1 high group compared to the KIR3DL1 low group. These results are in line with Boudreau et al. (2016) showing that KIR3DL1 subtypes exhibit distinct expression

patterns on the NK cell surface. However, the KIR3DL1 frequency was not associated with HCV infection outcome.

4.2.5 HLA-Bw4 copy number is associated with NK cell functionality in healthy individuals

Since the genetic analysis revealed a significant association between Bw4 copy number and infection outcome, as PWID encoding two or more Bw4 motifs were enriched in the anti-HCV seronegative group, a possible functional impact of a Bw4 copy number effect was addressed. Therefore, 90 PWID were grouped according to their number of Bw4 motifs rather than HCV infection status. A maximum of four Bw4 motifs can be expressed in a single individual on HLA-A and HLA-B alleles but since three as well as four Bw4 motifs are rarely seen, PWID were divided into three groups (0 Bw4, 1 Bw4 and patients with two or more Bw4 motifs). Functionality of KIR3DL1$^+$ NK cells was determined in response to stimulation with a HLA devoid target cell line K562 at an effector target ratio of 1:10. Differences in the ability to produce IFNγ and TNFα as well as in the degranulation potential was analysed.

Even though a slight tendency towards increased functional responses of KIR3DL1$^+$ NK cells could be observed with increasing Bw4 copy numbers, no significant differences between groups could be determined for any of the three analysed NK cell functions (figure 4.17 A-C). Since Kim et al. (2008) previously reported enhanced IFNγ responsiveness of KIR3DL1$^+$ NK cells in healthy individuals with multiple Bw4 copies and we could observe a trend in our PWID cohort as well, the aim was to validate our findings in a healthy cohort. Therefore, PBMCs from 120 healthy individuals were isolated and IFNγ, TNFα production and CD107a expression was analysed on KIR3DL1$^+$ NK cells as described for the PWID cohort. In line with previous results we observed a significant stepwise increase in IFNγ production with an increasing number of Bw4 motifs (figure 4.17 D). Moreover, the degranulation ability of KIR3DL1$^+$ NK cells was significantly increased in patients with one Bw4 motif compared to patients with no Bw4 motif and even more pronounced in patients that harbour two or more Bw4 copies. In addition, TNFα production was significantly higher in healthy individuals with two or more Bw4 motifs than in individuals with no or one Bw4 motif.

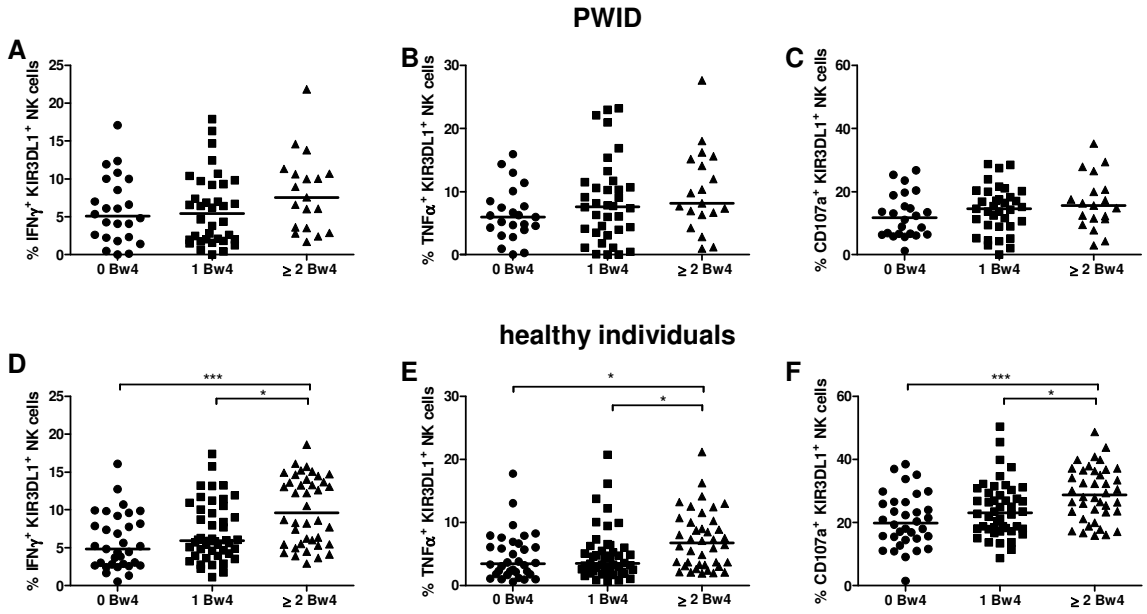

Figure 4.17: Correlation of HLA-Bw4 copy number and NK cell functionality in PWID and healthy individuals

PWID (A-C) and healthy individuals (D-F) were grouped according to the number of HLA-Bw4 alleles. Individuals lacking a Bw4 motif, with one Bw4 motif and individuals with two or more Bw4 motifs were grouped as indicated. The frequency of IFNγ producing (A+D), TNFα-producing (B+E) and CD107a (C+F) expressing KIR3DL1+ NK cells according to the number of Bw4 motifs are shown. Horizontal lines indicate the median. For statistical analysis Kruskal Wallis test or One-way ANOVA (*p≤ 0.05 and ***p≤0.001) were performed.

4.2.6 HLA-Bw4 80(T) is associated with an increased functional ability of NK cells in PWID

The genetic association studies revealed that the combination of HLA-Bw4 80(T) and KIR3DL1 is enriched in HCV-RNA negative PWID. Since this was the first report suggesting a protective effect of HLA-Bw4 80(T) in HCV infection, hereafter the influence of this genetic constellation on NK cell function was examined. To exclude effects possibly mediated by HLA-Bw4 copy number as stated in table 4.4 only PWID carrying a single HLA-Bw4 motif were analysed. Therefore, PWID with either one Bw4 80(T) or one Bw4 80(I) motif were compared and individuals lacking Bw4 were included as a control group. Interestingly, KIR3DL1+ NK cells of PWID expressing a Bw4 80(T) motif produced significantly higher amounts of IFNγ (p≤0.05) and TNFα (p≤0.01) and showed an enhanced degranulation ability (CD107a p≤0.05) compared to patients that harbour a Bw4 80(I) motif (figure 4.18 A-C). In addition, PWID encoding Bw4 80(T) expressed significantly more TNFα (p≤0.05) and CD107a (p≤0.05) than patients lacking a Bw4 motif. Furthermore, to address if this is a general or an HCV mediated effect, healthy individuals were grouped accordingly.

However, the increased functionality of KIR3DL1$^+$ NK cells in the context of HLA-Bw4 80(T) in PWID was not observed in healthy individuals (figure 4.18 D-F). No difference between individuals encoding a Bw4 80(I) motif or a Bw4 80(T) motif regarding IFNγ, TNFα and CD107a expression was detected.

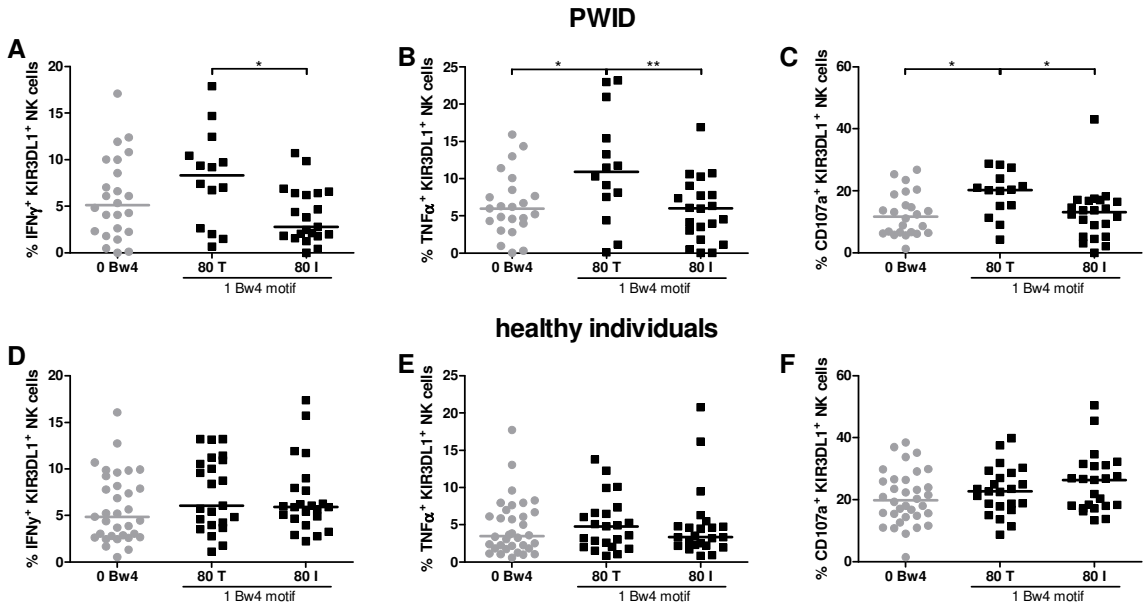

Figure 4.18: Functional analysis of KIR3DL1$^+$ NK cells from PWID and healthy individuals in the presence of the KIR3DL1 ligand HLA-Bw4 80(T) and 80(I)

PWID and healthy individuals were grouped according to their KIR3DL1-ligand status. PWID (A-C) and healthy individuals (D-F) carrying either no Bw4 or a single HLA-Bw4 80(T) or HLA-Bw4 80(I) motif were grouped as indicated. All individuals with multiple HLA-Bw4 alleles were removed from the analysis. The frequencies of IFNγ producing (A+D), TNFα producing (B+E) and CD107a expressing (C+F) KIR3DL1$^+$ NK cells according to the KIR3DL1-ligand status are shown. Horizontal lines indicate the median. For statistical analysis Kruskal Wallis test or One-way ANOVA (*p≤ 0.05 and **p≤ 0.01) were performed.

4.3 DNA methylation of the *IFNG* locus

This study described that Bw4 copy number is associated with enhanced NK cell functionality. To determine possible underlying mechanisms leading to improved IFNγ production of KIR3DL1$^+$ NK cells in individuals encoding multiple Bw4 motifs epigenetic alterations were analysed. Therefore, we aimed to investigate if Bw6$^+$ individuals showed an aberrant methylation pattern at the *IFNG* locus in comparison to individuals with two Bw4 motifs. Since epigenetic modifications of regulatory elements upstream or downstream of *IFNG* as well as methylation of the *IFNG* promoter can alter *IFNG* transcription (Melvin et al. 1995, Schoenborn et al. 2007), the DNA methylation pattern of the *IFNG* promoter as well as the upstream *CNS1* region was investigated. *CNS1* is located 4.2 kb upstream of the *IFNG* gene and displays a transcription factor binding site for NFAT and Tbet, thus is an enhancer for IFNG transcription (Lee et al. 2004).

4.3.1 DNA methylation analysis of the *IFNG* locus of naïve CD4$^+$ T cells, TH1 cells and NK cells

It has been reported that different cell types are associated with distinct methylation patterns of the *IFNG* locus (Dong et al. 2013). As a first step, we tested if the described methylation patterns could be reproduced in our study. Therefore, we determined the DNA methylation pattern of six CpG sites in the *CNS1* region and five CpGs in the *IFNG* promoter of naïve CD4$^+$ T cells, TH1 cells and NK cells in a healthy individual. Figure 4.19 displays a representative gating strategy used to sort naïve CD4$^+$ T cells and TH1 cells.

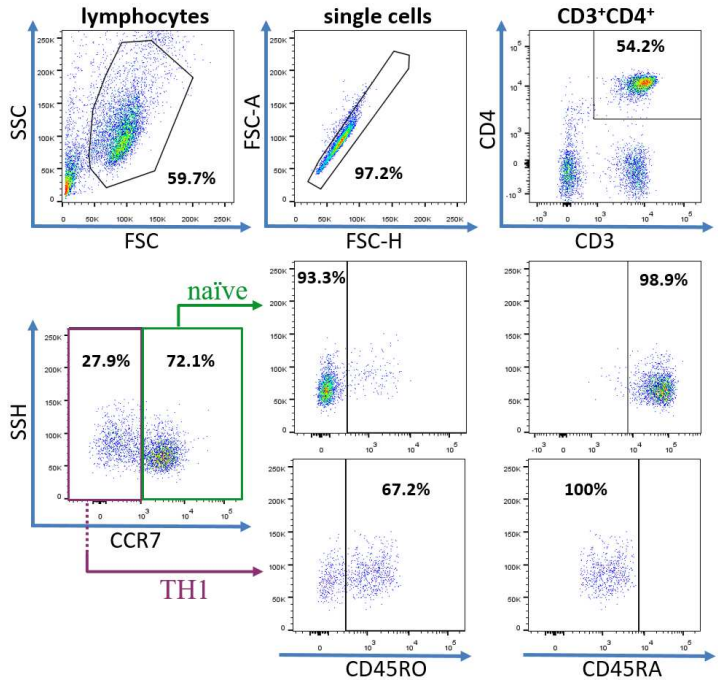

Figure 4.19: Representative gating strategy of FACS sorted naïve CD4⁺ T cells and TH1 cells

Freshly thawed PBMCs were FACS sorted for naïve CD4⁺ T cells and TH1 cells. Therefore, single lymphocytes expressing CD3⁺ and CD4⁺ cell surface antigens were further divided into CCR7⁺ CD45RO⁻ CD45RA⁺ naïve CD4⁺ T cells as well as CCR7⁻ CD45RO⁺ CD45RA⁻ TH1 cells.

While all analysed CpG sites were completely methylated in naïve CD4 T cells, TH1 cells showed a partial demethylation in the CNS region as well as at the *IFNG* promoter. NK cells displayed a demethylated *IFNG* promoter whereas the upstream element regulating *IFNG* transcription appeared to be highly methylated as described by Luetke-Eversloh et al. (2014). Hence, previous findings were reproduced and this method was utilised for further NK cell methylation analysis (figure 4.20).

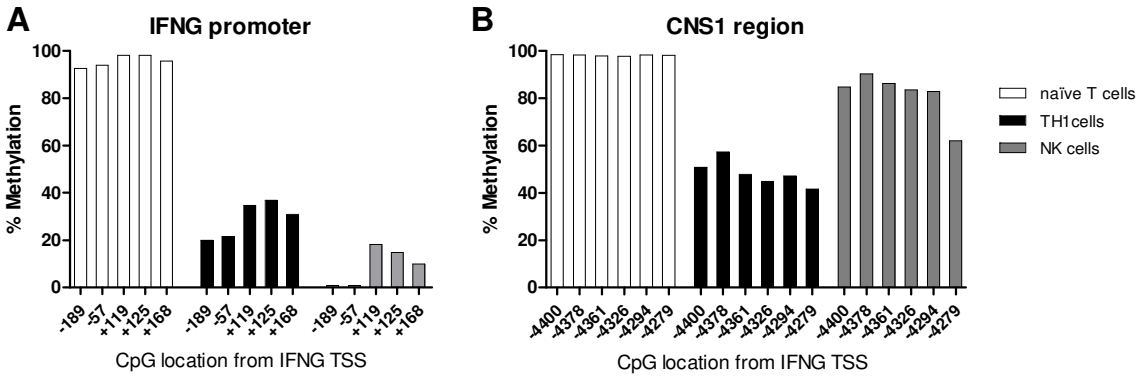

Figure 4.20: DNA methylation pattern of the *IFNG* locus from CD4⁺ T cells, TH1 cells and NK cells

The percentage of DNA methylation was determined via NGS for five CpG sites in the *IFNG* promoter (A) and six CpG sites in the *CNS1* region upstream of the *IFNG* promoter. Depicted is the pattern of FACS sorted naïve CD4 T cells (white), TH1 cells (black) and NK cells (grey) from one healthy individual. Furthermore, the distance of each CpG to the IFNG transcriptional start site (TSS) is indicated.

4.3.2 HLA-Bw4 copy number has no impact on *CNS1* methylation of NK cells

Since the detected results were in line with previous data it was further investigated if DNA methylation at the *IFNG* locus accounts for the observed difference in functionality of KIR3DL1$^+$ NK cells between Bw4$^+$ and Bw6$^+$ individuals. We hypothesised that individuals encoding multiple Bw4 motifs might show demethylation of the *CNS1* region resulting in higher transcription, leading to enhanced IFNγ production capability. Based on the observed complete demethylation of the *IFNG* promoter in NK cells the following analysis focused on the methylation pattern of the *CNS1* region. To exclude described differences in the methylation status between different NK cell developmental stages only CD56^{dim} NK cells were included (Luetke-Eversloh et al. 2014). KIR3DL1$^-$ and KIR3DL1$^+$ NK cells were sorted from ten healthy individuals including five individuals with two Bw4 motifs and five lacking a Bw4 motif. The gating strategy and representative dot plots are shown in figure 4.21.

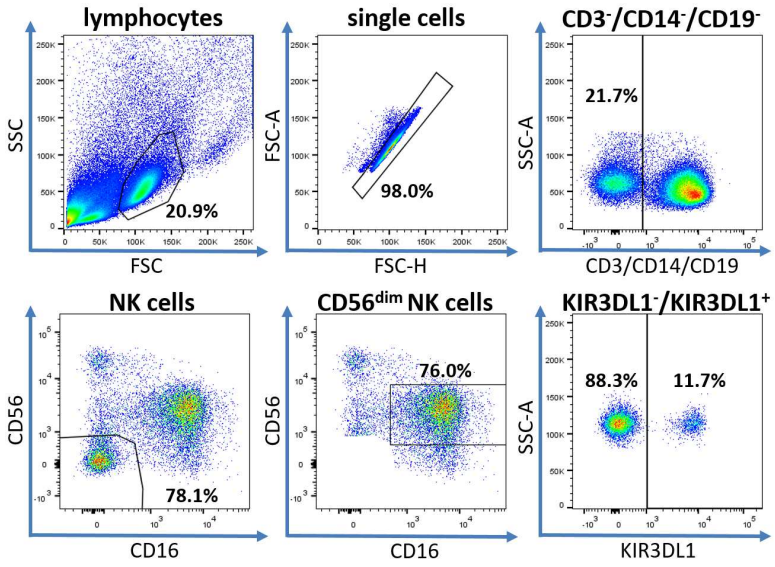

Figure 4.21: Representative gating strategy of FACS sorted KIR3DL1$^-$ and KIR3DL1$^+$ NK cells

PBMCs from 10 healthy individuals were FACS sorted for KIR3DL1$^-$ and KIR3DL1$^+$ CD56^{dim} NK cells. Cryopreserved PBMCs were thawed and NK cells were defined through CD16 and CD56 expression of CD3$^-$/CD14$^-$/CD19$^-$, live, single lymphocytes.

The level of methylation was analysed for each CpG site in the *CNS1* region. However, comparison between individuals expressing two Bw4 motifs or lacking a Bw4 motif revealed no significant differences, neither for KIR3DL1$^-$ nor KIR3DL1$^+$ NK cells (figure 4.22 A+B). Moreover, the mean methylation of all investigated CpG sites was similar between KIR3DL1$^-$ and KIR3DL1$^+$ NK cells irrespective of the number of Bw4 motifs (figure 4.22 C+D). This suggests that the number of Bw4 motifs is not

associated with an aberrant DNA methylation pattern of the *IFNG* locus in KIR3DL1$^+$ NK cells. While most samples showed a high level of methylation in the *CNS1* region, three samples were hypomethylated at the analysed six CpG sites (figure 4.22 D indicated in blue).

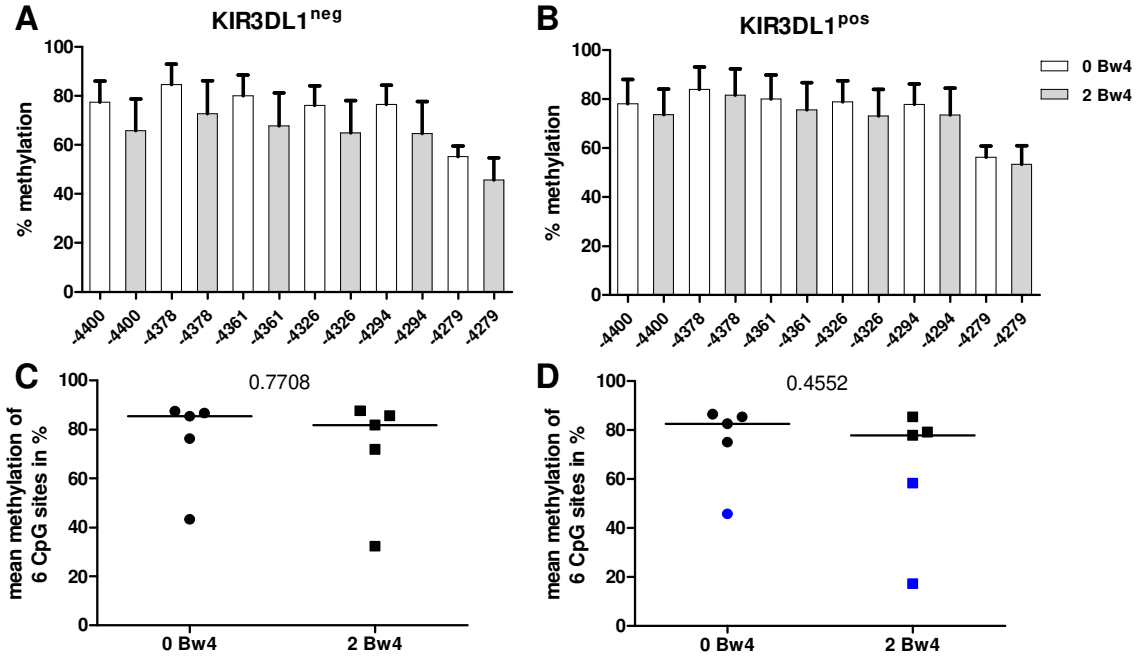

Figure 4.22: *CNS1* methylation pattern of KIR3DL1^{neg} and KIR3DL1^{pos} NK cells grouped by number of Bw4 motifs

KIR3DL1$^-$ and KIR3DL1$^+$ CD56^{dim} NK cells were sorted from five individuals without a Bw4 motif and five with two Bw4 copies. DNA methylation is depicted in percent for each of the six analysed CpG sites comparing 0 Bw4 with two Bw4 copies (A+B) and the mean with SEM is shown. The mean methylation of all CpG sites for every individual is depicted for KIR3DL1$^-$ (C) and for KIR3DL1$^+$ (D) NK cells. For statistical analysis, unpaired t-test was performed.

4.3.3 CMV seropositivity and NKG2C expression are associated with demethylation of the *CNS1* region of NK cells

It has been reported that the CMV status can influence the methylation pattern of NK cells (Luetke-Eversloh et al. 2014). Therefore, it was addressed if CMV seropositivity has an influence on the methylation pattern of KIR3DL1$^-$ and KIR3DL1$^+$ NK cells and thus accounts for the outliers seen in figure 4.22. Healthy individuals were grouped according to their CMV IgG status into CMV$^+$ and CMV$^-$ individuals. CMV$^+$ individuals showed a significantly lower mean methylation in comparison to CMV$^-$ individuals on KIR3DL1$^-$ ($p=0.0362$) as well as KIR3DL1$^+$ ($p=0.0483$) NK cells (figure 4.23 B). DNA demethylation in CMV$^+$ individuals was independent of KIR3DL1 expression on NK cells and could also be observed at each analysed CpG site individually (figure 4.23 A).

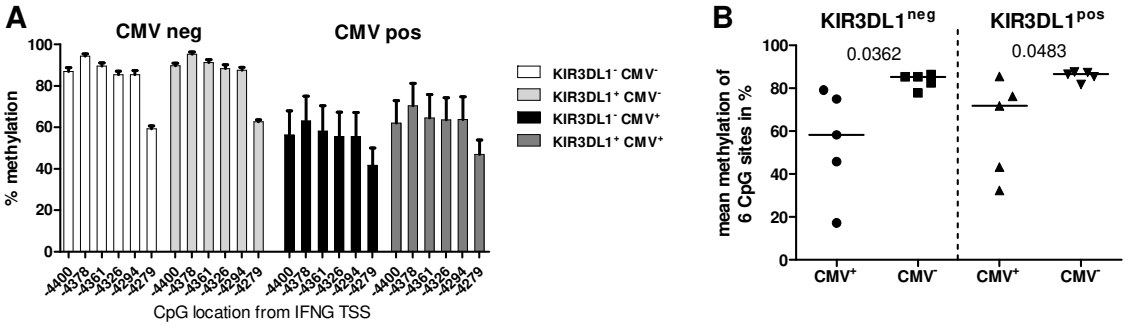

Figure 4.23: *CNS1* methylation pattern of KIR3DL1^{neg} and KIR3DL1^{pos} NK cells grouped by CMV status

The DNA methylation data from KIR3DL1$^-$ and KIR3DL1$^+$ NK cells of 10 healthy individuals was grouped according to the CMV status. The methylation of the *CNS1* region is depicted for each of the six analysed CpG sites (A). Furthermore, the mean methylation of the six measured CpG sites was calculated for each individual (B). P-values were calculated by unpaired t-test and the median is shown.

To further address the impact of the CMV status on DNA demethylation of the *CNS1* region, PBMCs were analysed by flow cytometry. As CMV seropositivity has been repeatedly linked to the expansion of NKG2C$^+$ NK cells and decreased DNA methylation of the *IFNG* locus has been described in CMV$^+$ individuals with an expansion of a NKG2C^{high} NK cell population, PBMCs were analysed for their NKG2C expression on NK cells (Luetke-Eversloh et al. 2014). In our study, a tendency towards an expansion of NKG2C$^+$ NK cells could be observed in CMV$^+$ individuals (figure 4.24 C). While three out of five examined CMV$^+$ individuals showed an NKG2C expansion, two individuals displayed normal frequencies of NKG2C$^+$ NK cells. The *CNS1* region of CMV$^+$ individuals without an expansion was highly methylated in KIR3DL1$^-$ and KIR3DL1$^+$ NK cells (figure 4.24 A+D) as seen in CMV$^-$ individuals. In contrast, hypomethylation of CpG sites in the *CNS1* region occurred in CMV$^+$ individuals with an expanded NKG2C population (figure 4.24 B+E). Interestingly, the mean methylation of all analysed CpGs significantly correlated with the expression of NKG2C on NK cells (figure 4.24 F, $p \leq 0.0001$ $R^2=0.8966$).

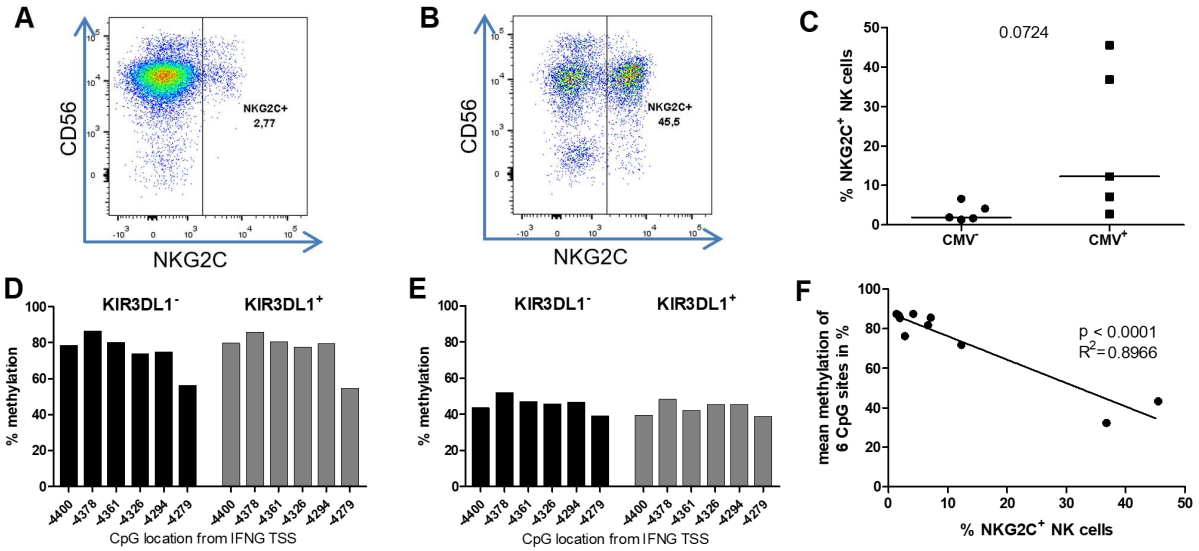

Figure 4.24: Demethylation of the *CNS1* region in CMV⁺ individuals with an NKG2C expansion

PBMCs from ten healthy individuals were analysed for their expansion of NKG2C⁺ NK cells. Cells were gated for single, viable lymphocytes and after exclusion of B cells, T cells and monocytes through CD3, CD19 and CD14, NK cells were defined by the expression of the cell surface antigens CD16 and CD56. A and B show exemplary flow cytometry plots from two CMV⁺ individuals either without (A) or with an expansion of the NKG2C population (B). Moreover, the corresponding methylation pattern of the *CNS1* region for both individuals is displayed in D and E. The percentage of NKG2C⁺ NK cells from all CMV⁻ and CMV⁺ individuals is depicted in C. Unpaired t-test was calculated to determine significance between groups. The frequency of NKG2C⁺ NK cells was correlated with the mean methylation of the six investigated CpG sites in the *CNS1* region and a linear regression analysis was performed.

5 Discussion

The importance of studying HCV infection is highlighted by the fact that the 2016 Lasker award went to Rice, Bartenschlager and Sofia for their contributions in the field of HCV infection research. Rice and Bartenschlager developed a replication system that enabled high throughput screening of novel HCV drugs (Lohmann et al. 1999, Lindenbach et al. 2005) and Sofia was awarded for the discovery of Sofosbuvir a highly efficient NS5B nucleotide polymerase inhibitor (Sofia et al. 2010). Nevertheless, the underlying mechanisms that either confer protection prior to seroconversion or lead to spontaneous resolution of HCV infection are not fully defined. Therefore, the influence of NK cells and iNKT cells on spontaneous resolution, protection from HCV infection or their role in chronic HCV infection were further addressed in this thesis.

5.1 iNKT cells in HCV infection

CD1d restricted iNKT cells, one subset of NKT cells, have been repeatedly reported to play a role in various viral infections (De Santo et al. 2008, Fernandez et al. 2014, Li et al. 2016, Ahmad et al. 2017). However, the involvement of iNKT cells in HCV infection remains poorly understood and their contribution to early immune responses as well as to liver injury in humans is not well defined. Therefore, the aim of this study was to comparatively analyse the phenotype and function of iNKT cells in HCV infected PWID with different infection outcome in detail. The role of iNKT cells in HCV infection was determined in a high-risk cohort of anti-HCV positive PWID. The cohort was grouped into PWID with detectable HCV-RNA (HCV-RNA positive) and anti-HCV seropositive individuals without detectable RNA (HCV-RNA negative). The comparison of the phenotype of iNKT cells from HCV-RNA positive and HCV-RNA negative PWID suggested iNKT cells to be activated in chronic HCV infection as they showed significantly increased expression of the activation marker CD38. Expression of various exhaustion and differentiation markers as well as NK cell receptors however, did not correlate with HCV infection outcome. Moreover, iNKT cell frequencies as well as their functionality were similar between PWID groups. However, iNKT cells from HCV-RNA positive PWID showed a trend towards an increased proliferation potential as well as an enhanced IFNγ production capacity. *In vitro* expansion of iNKT cells resulted in a significant upregulation of CD38

independent of HCV infection outcome. Interestingly, cytokines associated with NK cell activation significantly activated iNKT cells as measured by an increase of CD38 expression. Notably, the frequency of iNKT cells in the liver was not enriched compared to frequencies in the peripheral blood, however, we were unable to test liver samples from HCV-infected individuals, as nowadays liver biopsies to determine the stage of liver fibrosis in HCV infected individuals are replaced by non-invasive approaches (reviewed in Castera 2012). It is well described, that IFN levels and consequently ISG expression levels are elevated in the liver of patients with chronic infection (Helbig et al. 2005, Sarasin-Filipowicz et al. 2008). We therefore tested, if treatment of peripheral iNKT cells with IFNα or IFNλ resulted in iNKT cell activation and thus an upregulation of CD38. Although such a direct effect on peripheral iNKT cells was not observed, an increase of CD1d expression was noted on monocytes, which is the cell type with the highest CD1d expression in the periphery. It would be important to analyse the impact of IFN treatment on CD1d expression levels on liver resident cells and more importantly to directly address if CD1d expression levels are elevated in the liver compartment in the context of HCV infection. In a cooperation project, the latter aspect was addressed and expression profiles from liver biopsies of HCV and HBV infected livers were compared. In line with an increased expression of CD1d in the context of chronic HCV infection in the liver, CD1d transcript levels were significantly higher in the HCV-infected liver compared to the HBV-infected liver. Although not conclusive, this could point towards a role of type I or type III interferons in intrahepatic CD1d regulation, because it has been described that ISGs are strongly upregulated in the liver during hepatitis C but not hepatitis B (Su et al. 2002, Wieland et al. 2004).

iNKT cells have been extensively studied in mice models and associated with a role in several viral infections such as MCMV, HBV and HSV. iNKT cell activation resulted in reduced (van Dommelen et al. 2003) or inhibited (Kakimi et al. 2000) viral replication of MCMV and HBV, mediated by the induction of IFNγ, suggesting that in addition to NK cells NKT cells can mediate early viral clearance by either directly killing the virus infected cells or indirectly activating other immune mechanisms. Furthermore, the absence of iNKT cells was associated with impaired clearance of HSV (Grubor-Bauk et al. 2003). In addition, there are multiple indications in hepatitis mouse models for the importance of iNKT cells in liver immunology. Moreover,

mouse models could provide explanations for the importance of iNKT cells in the clearance or the progression of HCV infection. Previous studies proposed that activated iNKT cells can mediate hepatocyte damage (Osman et al. 2000) even in the absence of NK cells (Nakagawa et al. 2001). In addition, it has been reported that iNKT cell activation contributes to the development of liver fibrosis (Ishikawa et al. 2011). Furthermore, IFNγ produced by activated iNKT cells enhanced NK cell cytotoxicity as well as potentiated their IFNγ capacity and increased $CD8^+$ T cell responses (Carnaud et al. 1999, Nakagawa et al. 2001). Collectively, the existing mouse data suggests that iNKT cell activation in acute HCV infection might be beneficial for clearance, as they activate NK as well as T cells which are important mediators for early immune control of HCV infection. Whereas, activated iNKT cells might be disadvantageous in chronic infection as they have the ability to promote liver fibrosis. Based on data generated in various mouse models we hypothesised that potentially iNKT cells play a role in HCV infection outcome. However, there might be some limitations in the transferability into the human system. Although some similarities between CD1d restricted iNKT cells from mice and humans have been described, there are major differences as well. Especially reported iNKT cell frequencies in several tissues differ vastly between these species. Whereas high frequencies of CD1d restricted iNKT cells have been described in livers of mice (approximately 30% of IHL), invariant $V\alpha24^+$ iNKT cells were rare in the human liver (0.03-0.34% of IHL) (Matsuda et al. 2000, Exley et al. 2002). Our report proposed even lower iNKT cell frequencies in the human liver (mean: 0.03% of IHL). Furthermore, in accordance with our study, iNKT cell frequencies in the peripheral blood of humans were reported to be highly variable compared to mice and span a 100-fold range in healthy individuals (Lee et al. 2002). Therefore, suggesting that mice studies might not adequately reflect the importance of iNKT cells in human HCV infection. Even though hepatitis mouse models have provided evidence for the role of iNKT cells in liver inflammation (Ishikawa et al. 2011) and the recently developed HCV mouse model (Billerbeck et al. 2017) offers the opportunity to study iNKT as well as non-invariant NKT cells in acute and chronic infection, these models cannot replace human cohort studies.

In HCV infection, no alterations in iNKT cell numbers have been reported upon treatment with PEGylated IFNα and ribavirin. Moreover, iNKT cell frequencies were

neither associated with HCV genotype nor with SVR to IFNα based treatment (van der Vliet et al. 2005). Thus, this indicates that iNKT cell frequencies were not influenced by HCV therapy and moreover did not predict treatment response. Here, however we aimed to elucidate the role of iNKT cells on HCV infection outcome rather than treatment outcome. Therefore, the underlying study solely focused on iNKT cell analysis in treatment naïve HCV infected patients. Our study described no differences in iNKT cell frequencies in HCV-RNA positive and HCV-RNA negative PWID, thus our results were in accordance with studies from Inoue et al. (2006) and van der Vliet et al. (2005) that reported comparable Vα24$^+$ Vß11$^+$ iNKT cells frequencies in the periphery. However, Lucas et al. (2003) provided conflicting results, as they described decreased frequencies of Vα24$^+$ Vß11$^+$ iNKT cells in HCV-RNA positive individuals compared to HCV-RNA negative individuals with resolved HCV infection. Since no anti-HCV negative PWID were included in our iNKT cell study, we were not able to confirm reported elevated iNKT cell frequencies in exposed uninfected individuals (Werner et al. 2013). The explanation for the reported differences in iNKT cell frequencies between cohorts is likely to be multifactorial. However, differences in definition of the iNKT cell population through varying methodology could be excluded as the underlying cause. All included studies fulfilled the criteria of a clearly defined iNKT cell population, since staining was performed for the Vα as well as the Vβ chain of the invariant TCR and not solely through the expression of the cell surface markers CD56 and CD3. Although the iNKT cell definition in our study differed, as iNKT cell flow cytometric analysis was based on the ability of iNKT cells to recognise αGalCer loaded CD1d dextramers, we provided evidence that the definition of iNKT cells through the TCR chains Vα24 and Vß11 was congruent with the dextramer analysis. Thus, investigated iNKT cell populations were comparable based on the methodology used for their definition. In addition, various characteristics such as gender and age have been described to affect iNKT cell numbers. Sandberg et al. (2003) provided evidence that the female sex was associated with elevated Vα24$^+$ iNKT cell frequencies. Moreover, an inverse correlation between iNKT cell frequencies and age has been proposed in healthy individuals (Mansour et al. 2015). Although our association analysis was limited in regard of gender evaluation, since the proportion of male PWID was significantly higher than female PWID in our cohort, a weak association between iNKT cell frequencies and age in HCV infected individuals was observed in our study as well.

In HCV infection, the female sex that has been linked to increased iNKT cell frequencies, has a higher likelihood for viral clearance compared with males (Bakr et al. 2006), thus gender distribution in HCV cohorts is generally different between the HCV-RNA positive group and the HCV-RNA negative group. Variations in gender distribution might partially explain discrepant results between cohorts. In our iNKT cell study the mean age in years as well as the gender distribution of PWID was comparable between HCV-RNA positive and HCV-RNA negative individuals. However, other studies might not be well controlled for age and gender and could account for the observed differences.

Interestingly, iNKT cells of HCV-RNA positive PWID showed significantly higher expression of the activation marker CD38 than HCV-RNA negative PWID. Although iNKT cells have not been associated with an activated phenotype in the periphery before, Lucas et al. (2003) reported that liver Vα24$^+$ Vß11$^+$ iNKT cells of HCV infected individuals compared to peripheral iNKT cells displayed an elevated expression of CD69, a marker for recent activation. Moreover, liver infiltrating NKT cells from chronically HCV infected individuals, defined as CD56$^+$CD3$^+$ lymphocytes, showed a trend towards increased CD69 expression compared to HCV negative patients (Boisvert et al. 2003). In addition, activation of mucosal associated invariant T (MAIT) cells, another population of semi-invariant T cells which displays several similarities with NKT cells (reviewed in Treiner et al. 2006) has been reported in chronic HCV infection (van Wilgenburg et al. 2016). Taken together these data support our findings that iNKT cells are activated during chronic infection. However, longitudinal studies are needed to determine if this activation state is induced by HCV infection or if elevated frequencies of CD38$^+$ iNKT cells promote persistence of HCV infection. Despite the activated phenotype of iNKT cells from chronically HCV infected individuals, none of the other analysed markers were associated with infection outcome. Although functional impairment of iNKT cells has been linked to enhanced PD1 expression in HIV infected individuals (Moll et al. 2009) and exhaustion of HCV specific CD8$^+$ T cells in chronic infection were commonly characterised by an increased PD1 expression (reviewed in Timm et al. 2015), PD1 expression levels on iNKT cells were neither associated with HCV infection outcome nor reduced functional capacity.

While the enhanced *ex vivo* activation, seen in chronically infected individuals was not linked to the extent of iNKT cell activation after *in vitro* expansion, expansion upon αGalCer stimulation was in general associated with a significant upregulation of CD38 on the cell surface. Our reported phenotype of iNKT cells after proliferation was expected and in line with a study by Kitamura et al. (2000) who described an upregulation of the early activation marker CD69 on iNKT cells upon αGalCer stimulation. Therefore, we propose that the expansion of iNKT cells in HCV infected individuals is associated with tremendous activation of iNKT cells.

As αGalCer is an exogenous antigen derived from a marine sponge, iNKT cell activation in HCV infection is mediated by other mechanisms. Critical factors that regulate activation and effector functions of iNKT cells are the type of antigen-presenting cell, the availability of the presented antigen and the cytokine environment. Possibly, the cytokine milieu in chronic HCV infection influences iNKT cells and might activate them which could explain the observed CD38 upregulation. To provide insight into the underlying mechanisms the influence of various cytokines previously described to be associated with HCV infection or NK cell activation was analysed in this study. Our study demonstrated that IFNα treatment indirectly and a combination of IL12, -15 and -18 directly activated iNKT cells. IFNα based therapy of chronically HCV infected individuals was described to not alter MAIT cell frequencies but to enhance CD38 expression on MAIT cells (Spaan et al. 2016). In contrast, no direct effect of IFNα treatment on the frequency of $CD38^+$ iNKT cells was observed in our study. However, van Wilgenburg et al. (2016) described that the activation of MAIT cells by IFNα was dose dependent. MAIT cell activation was only induced with particularly high IFNα concentrations that far exceeded the concentration used in our study. This might explain the absence of a direct effect of IFNα treatment on CD38 expression on iNKT cells in our analysis. In line with our report suggesting a direct effect of the combination of IL12, -15 and -18, the early activation marker CD69 was significantly upregulated on MAIT cells upon IL12 and IL18 stimulation (Spaan et al. 2016). The activation of MAIT cells was dose dependent and the combination of IL12 and IL15 was sufficient to induce a high activation state (van Wilgenburg et al. 2016). Since IL18 alone had no impact on CD38 expression, we as well propose that a combination of IL12 and IL15 is sufficient to activate iNKT cells. Elevated IL12 serum levels have been described in HCV infection and IFNγ priming induced a significant increase of IL12 in chronically HCV infected individuals especially in patients with

severe liver inflammation (Quiroga et al. 1998). In addition, serum IL15 was also significantly elevated in chronic HCV infection, compared to healthy controls and increased with disease progression to HCC (Kakumu et al. 1997). Collectively, the existing data support our idea that IL12 and IL15 probably influence iNKT cell activation in the natural course of HCV infection and might explain the activated phenotype of iNKT cells in chronically HCV infected individuals.

iNKT cells are activated either by direct or indirect mechanisms. The direct mechanisms via a strong TCR signal would include the specific recognition of a viral or virus-induced antigen. However, no HCV derived glycolipid that can activate either iNKT or non-invariant NKT cells via CD1d presentation has been identified so far. Indirect mechanisms by virus induced cytokines combined with a weak TCR signal mediated by an endogenous glycolipid are more likely to induce iNKT cell activation in the HCV infection setting. Even though iNKT cells in HCV infection probably depend only on a weak TCR signal, antigen presentation by CD1d is still crucial for iNKT cell activation. It has been demonstrated that viruses can manipulate CD1d expression to escape recognition by CD1d restricted iNKT cells, thus leading to reduced iNKT cell activation. HCMV, HSV and HIV developed mechanisms to downregulate CD1d surface expression through inhibition of CD1d transcription, increasing the internalisation of CD1d, intracellular retention of CD1d molecules and active prevention of the reappearance of CD1d on the cell surface after endocytosis (Chen et al. 2006, Yuan et al. 2006, Raftery et al. 2008, Han et al. 2013). Our study however reported no differences in CD1d expression on monocytes as well as B cells between HCV-RNA positive and HCV-RNA negative PWID in the periphery, indicating that in HCV infection CD1d expression is not altered on peripheral cells. Interestingly, CD1d mRNA levels in the liver were significantly higher in HCV infected patients compared to HBV infected patients. In contrast to CMV, HSV and HIV that downregulate CD1d expression, CD1d expression seems to be upregulated in HCV infected livers and therefore might explain the activated phenotype of iNKT cells in chronically infected PWID, as upregulation of the ligand CD1d in chronic HCV infection can subsequently lead to iNKT cell activation. Upregulation of CD1d might not only mediate iNKT activation but could contribute to non-invariant NKT cell activation as well, since this subset is also CD1d restricted. IFNα treatment of CD1d expressing monocytes significantly enhanced CD1d surface expression and might explain elevated CD1d mRNA levels in the liver from HCV infected individuals. Taken

together these data suggest that continuous exposure to IFNs in chronically HCV infected individuals might result in iNKT cell activation. In addition, another mechanism that might account for the activation of iNKT cells in the HCV infection setting besides the upregulation of the ligand CD1d or the upregulation of cytokines could be the induction of the expression of CD1d restricted endogenous lipid antigens as seen in HBV infection (Zeissig et al. 2012). However, such mechanisms have not been reported for HCV infection so far.

This study provides evidence for the activation of iNKT cells in chronic HCV infection and proposes two possible mechanisms that might mediate this activation. However, the functional impact of this activation has been inconclusive and needs to be further addressed in future studies. Interestingly, in comparison to the previously described low proliferation potential of Vα24$^+$ Vß11$^+$ iNKT cells in HIV infection (Moll et al. 2009), our study demonstrated that iNKT cells in HCV infection rapidly proliferate after activation with αGalCer. In addition, even though not statistically significant a trend towards an increased proliferation capacity of iNKT cells of HCV-RNA positive individuals compared to HCV-RNA negative individuals was observed. Thus, the increased expansion of iNKT cells in chronic infection might indicate that the pre-activated state results in a reduced proliferation threshold. Furthermore, functional analysis of iNKT cells from HCV-RNA positive and HCV-RNA negative PWID, performed *ex vivo* as well as after *in vitro* expansion, revealed no association between the functional capacity of iNKT cells to produce IL2, IFNγ or their degranulation ability and infection outcome. However, a tendency towards increased IFNγ production was observed in chronically HCV infected individuals compared to patients that spontaneously resolve the infection. Although iNKT cells in the liver were reported to be mainly TH1 restricted, CD4$^+$, CD4$^-$ and DN iNKT cell subsets in the periphery each harbour distinct effector functions (Exley et al. 2002, Gumperz et al. 2002). As discussed earlier in this study iNKT cell frequencies were low in the periphery and moreover material from HCV infected PWID is limited. Therefore, sufficient numbers of iNKT cells to further subdivide them into TH1 and TH2 iNKT cell subsets could not be acquired. Hence, our analysis could not exclude the possibility that IFNγ production by TH1 iNKT cells in the periphery might significantly differ according to HCV infection outcome. Furthermore, differences in effector functions might have been overlooked as an artificial stimulation with PMA and Ionomycin was

used to induce iNKT cell activation. Thus, a more physiological stimulation, for instance with cytokines, might reflect the situation in HCV infection more accurately. Despite this, our study provided evidence that iNKT cells in the periphery are efficient IFNγ producers.

Although collectively the results indicate that chronic inflammation as observed in chronic HCV infection leads to activation of iNKT cells, our data are based on analysis of iNKT cells from the peripheral blood. It would be important to study iNKT cells at the site of infection, which is on the one hand in general technically challenging since only a few mononuclear cells can be isolated from liver biopsies and on the other hand, is nowadays highly limited by the number of biopsies available from HCV infected patients, since non-invasive strategies are preferred to determine the stage of liver fibrosis (Castera 2012). Moreover, acquisition of liver material through biopsies from patients with resolved infection is ethically challenging as there is generally no medical indication for such an invasive procedure. Currently, isolation of IHL from liver perfusions provides the opportunity to study, for instance iNKT cells at the site of HCV infection.

Notably, in line with Karadimitris et al. (2001) the frequency of iNKT cells in the liver was low and comparable to frequencies in the peripheral blood. However, contradicting data exists as significant enrichment of Vα24$^+$ Vβ 11$^+$ iNKT cells in the liver compartment compared to the periphery, has been reported in healthy individuals (Ward et al. 2004). One has to keep in mind that our approach to use IHL, obtained by liver perfusion, has limitations since material was obtained from patients undergoing liver resections with various underling diseases. Therefore, the comparison between iNKT cells in both compartments provided by Ward et al. (2004) might reflect the situation in healthy individuals more accurately as they analysed liver perfusate from transplanted healthy livers. However, we were unable to test liver samples from HCV-infected individuals in this study, thus could not address differences in iNKT cell frequencies and comment on a previously described trend towards ongoing reduction of Vα24$^+$ iNKT cell frequencies during HCV disease progression as well as depletion of Vα24$^+$ iNKT in patients with end-stage HCV cirrhosis (Deignan et al. 2002). Although we could not address this in the underlying study, a comparison of iNKT between both compartments could give further insights into the role of iNKT cells during HCV infection and might provide evidence for the

functional impact of the activated state observed in chronically HCV infected PWID. In addition, the described effect of IFNα on CD1d expression points towards a role of IFNs in hepatic CD1d regulation. However, with the analysis of monocytes from the periphery an artificial system was used that might not adequately reflect the situation in the liver compartment. Thus, to conclusively determine IFN mediated upregulation of CD1d, IHL as well as CD1d expressing hepatocytes have to be analysed. Although, in our study neither liver enzymes nor viral load of HCV-RNA positive individuals correlated with iNKT cells frequencies in the periphery, which is in accordance with previously reported MAIT cell data (Spaan et al. 2016), iNKT cells in the HCV infected liver might still be associated with hepatocyte damage. In addition to studying HCV infected liver samples, the use of a HCV cell culture replicon system might provide further insights into the possibly antiviral properties of human iNKT cells. Efficient suppression of HCV replication in a genotype 2a HCV cell culture replicon system by IFNγ production of activated MAIT, has recently been described (van Wilgenburg et al. 2016). Even though iNKT cells display a much smaller proportion of intrahepatic lymphocytes compared to MAIT cells, they have the ability to rapidly expand upon activation, thus might play a critical role in HCV infection.

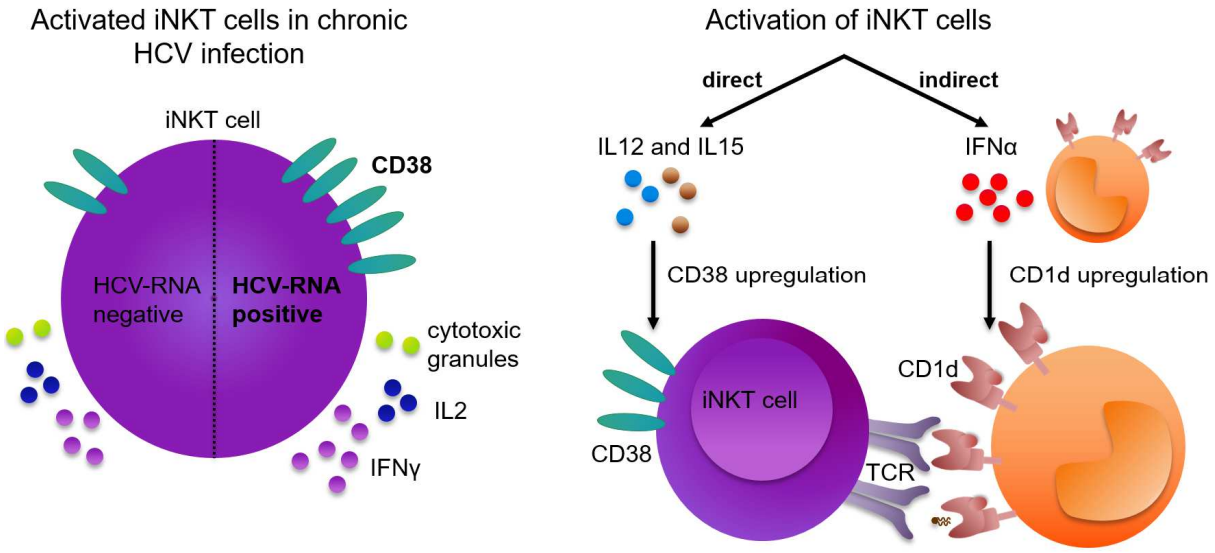

Figure 5.1: Graphical summary: activated iNKT cells in chronic HCV infection

iNKT cells are activated in HCV-RNA positive PWID. Two possible mechanisms have been postulated in this study that result in the direct or indirect activation of iNKT cells in HCV infection. Cytokines as IL12 and IL15 directly activate iNKT cells whereas IFNα leads to an indirect activation through the upregulation of CD1d, the ligand for iNKT cells. Activated iNKT cells of HCV RNA positive PWID might for instance contribute to hepatocyte damage during chronic HCV infection. Even though we are lacking human data this effect of activated iNKT cells has been described in mice. However, to conclusively link activated iNKT cells to HCV pathology further research is needed.

Collectively, our data provides evidence that iNKT cells from HCV-RNA positive PWID are activated in chronic HCV infection. In addition, two possible mechanisms have been proposed that result in the activation of iNKT cells in HCV infection. IFNα mediated upregulation of the iNKT cell ligand CD1d and the direct effect of IL12 and -15 on CD38 expression indicate that CD1d expressing cells and the cytokine environment modulate iNKT cell activation in HCV infection. In conclusion, additional studies are needed to elucidate the role of iNKT cells in the context of HCV infection further and to characterise the impact of the activation status on the functionality in more detail.

5.2 NK cells in HCV infection

This study was based on the recent finding of our group, that a distinct KIR/KIR ligand constellations was associated with HCV infection outcome in a high-risk cohort of PWID. HCV-treatment naïve patients were grouped according to their antibody serostatus and viral RNA levels into anti-HCV seropositive PWID with detectable HCV RNA, anti-HCV seropositive PWID without detectable HCV RNA and anti-HCV seronegative PWID. The genetic combination of KIR3DL1 and a HLA class I allele encoding a Bw4 80(T) motif was significantly enriched in HCV infected patients that spontaneously control the infection. This effect was not observed in the anti-HCV seronegative group. The association of KIR3DL1/HLA-Bw4 80(T) and spontaneous clearance of HCV infection was confirmed in a second independent cohort of PWID from North America. This indicates that NK cells in the context of KIR3DL1 combined with the ligand HLA-Bw4 80(T) are beneficial for the outcome of acute HCV infection mediating resolution of the virus but do not confer protection prior to seroconversion. Therefore, the influence of this genetic constellation on NK cell function was addressed and confirmed a superior functionality of KIR3DL1$^+$ NK cells from Bw4 80(T) positive patients compared to Bw4 80(I) positive patients. Moreover, a second distinct predisposition associated with infection outcome was identified in the genetic correlation study. KIR3DL1 in combination with multiple copies of the ligand HLA-Bw4 (≥ two Bw4 motifs) was significantly increased in individuals with an anti-HCV seronegative state. Therefore, this genetic constellation mediated a protective effect upon HCV exposure which results in resolution prior to seroconversion. In addition, functional analysis confirmed an enhanced cytokine secretion as well as cytotoxic potential of NK cells in individuals with multiple Bw4 copies. Hence this study proposes two distinct genetic predispositions that result in either protection from HCV acquisition prior to seroconversion or driving spontaneous resolution of HCV infection. Interestingly, both genetic associations were associated with enhanced NK cell functionality.

Here, we examined the human NK cell subset expressing KIR3DL1, the only known KIR specific for HLA-Bw4 alleles. KIR3DL1 frequencies on NK cells were similar between HCV-RNA positive, HCV-RNA negative and anti-HCV negative PWID in our cohort. In line with this study Alter et al. (2011) reported similar KIR3DL1 frequencies in HCV-RNA positive and HCV-RNA negative individuals. Moreover, in contrast to

previously described decreased proportions of KIR3DL1 expressing NK cells in HCV infected patients the observed expression of KIR3DL1 on NK cells from healthy individuals and chronically HCV infected individuals was comparable in our analysis (median frequency of KIR3DL1$^+$ NK cells 14.5% vs. 15.1%). Differences might be due to the low number of included individuals in the study of Oliviero et al. (2009) while our flow cytometric analysis included 90 PWID and 120 healthy individuals. Therefore, we propose that the frequency of KIR3DL1 expressing NK cells is not associated with HCV infection outcome in PWID.

However, KIR expression on T cells might influence HCV infection outcome. The frequency of KIR3DL1 expressing T cells from healthy individuals were low compared to NK cells. The frequencies of KIR3DL1$^+$ CD8$^+$ T cells ranged from 0.02% to 7.83% whereas KIR3DL1 was mostly undetectable on CD4$^+$ T cells (mean: 0.15%, appendix figure 6.3). In line with our results low levels of KIR (KIR2DL1/S1/L2/S2/L3) expression on CD8$^+$ T cells have been described in the periphery as well as in the liver from HCV infected individuals. Even though KIR expression on HCV-specific CD8$^+$ T cells in the liver was low, Bonorino et al. (2007) proposed that expressed KIRs are able to efficiently impair their virus specific reactivity. KIR expressing CD8$^+$ T cells in HCV infection were terminally differentiated and displayed a reduced replicative potential. Although KIR3DL1 expression on CD8$^+$ T cells was not further investigated in this study, existing data suggests that KIR expression on T cells might be disadvantageous for the adaptive immune response against HCV infection.

Several studies associated different KIR/KIR ligand constellations with disease severity or outcome in various virus infections such as HIV or HCV (Martin et al. 2002, Khakoo et al. 2004). Moreover, reduction of CMV reactivation after stem cell transplantation was correlated with the presence of multiple activating KIRs in the donor (Cook et al. 2006). In HCV infection, the genetic constellation of the inhibitory NK cell receptor KIR2DL3 in combination with homozygosity of the ligand HLA-C1 was reproducibly associated with infection outcome. Khakoo et al. (2004) were the first who identified a direct influence of KIR2DL3 and HLA-C1 on spontaneous resolution of HCV infection, which was confirmed in a PWID cohort of Puerto Rican ethnicity (Romero et al. 2008). Furthermore, the same group could extend the association of this genetic predisposition to protection from HCV infection prior to seroconversion (Zúñiga et al. 2009). In addition, Knapp et al. (2010) confirmed the

association with HCV resistance in seronegative PWID and further correlated homozygosity for KIR2DL3 in combination with HLA-C1 allotypes with an enhanced likelihood to achieve SVR upon IFNα-based therapy. In contrast, no correlation of this KIR/KIR ligand constellation with HCV infection outcome was detected in our genetic association study. However, a previous study of our group could show increased KIR2DL3 expression on NK cells of seronegative PWID and PWID with resolved infection compared to HCV-RNA positive PWID (Thöns et al. 2014). The discrepancy between results from genetic association studies might be explained by factors that influence HCV infection outcome such as viral genotype, gender and the IL28B genotype, since these factors are not always well controlled (Grebely et al. 2014). Moreover, HCV resolution is not solely mediated by NK cell responses but CTLs, especially virus specific $CD8^+$ T cells with a broad response efficiently clear the infection (Cooper et al. 1999). $CD8^+$ T cell responses in HCV infection are partially genetically determined. Immunogenetic studies reproducibly associated HLA-B*27 and HLA-B*57 with spontaneous immune control of HCV infection (McKiernan et al. 2004, Kim et al. 2011, Fitzmaurice et al. 2015). In addition, our group confirmed the protective effect of HLA-B*27 for PWID exposed to genotype 1 as well as genotype 3 (data unpublished). Moreover, the occurrence of the HLA-B*27:05 allele that comprises a Bw4 80(T) motif was associated with infection outcome in our PWID cohort. To exclude that the genetic correlation of KIR3DL1/HLA-Bw4 80(T) and spontaneous clearance of HCV infection in our PWID cohort was exclusively driven by HLA-B*27, all B*27 positive patients were excluded. The analysis showed that the protective effect of the KIR3DL1/HLA-Bw4 80(T) genotype was not solely mediated by HLA-B*27 since the frequency of this constellation was still higher in HCV-RNA negative PWID (37.5%) compared to HCV-RNA positive PWID (22.9%) however, this association was borderline significant ($p=0.05$). Although studies provided evidence that adaptive as well as innate immune events independently contribute to the outcome of HCV infection (Khakoo et al. 2004, McKiernan et al. 2004, Thomas et al. 2009), collectively our results suggest an additive effect of HLA-B*27 and KIR3DL1/HLA-Bw4 80(T) on HCV infection outcome.

The beneficial effect of the KIR3DL1/HLA-Bw4 80(T) genotype on spontaneous clearance of HCV infection could be demonstrated in two independent HCV cohorts. However, no association was observed in a third cohort from the UK. In addition to

the above stated factors, that could influence the observed variances between genetic association studies, the most probable explanation for the variance between the UK and our cohort are differences in distribution of HLA-B alleles between cohorts. Differences in HLA-B allele frequency as observed for HLA-B*13, *38, *44 and *57, might affect the contribution of CTLs to HCV resolution and therefore account for observed difference in the genetic association study.

Interestingly, we observed that in the context of HLA-Bw4 80(T) NK cell functionality was enhanced compared to HLA-Bw4 80(I) allele carriers in PWID, whereas this phenomenon was not observed in healthy individuals. The HLA-Bw4 subtype that harbours an isoleucine (I) at amino acid position 80 exhibits a stronger affinity to KIR3DL1 compared to threonine (T) at this position (Cella et al. 1994) and therefore should be better armed to efficiently produce cytokines upon MHC downregulation. This phenomenon was observed by Boudreau et al. (2016) in healthy donors, since KIR3DL1$^+$ NK cells with a HLA-Bw4 80(I) background exhibited a better functionality compared to HLA-Bw4 80(T) positive individuals against HLA-negative targets cells. In contrast, to these results we observed no functional benefit of HLA-Bw4 80(I) in healthy individuals. Furthermore, the reason for our observed superior functionality of KIR3DL1$^+$ NK from HLA-Bw4 80(T) positive PWID remains unclear. Previous data might provide an explanation for the reported superior functionality of KIR3DL1$^+$ NK cells in the context of a weaker affinity ligand as HLA-Bw4 80(T) in HCV infection. KIR2DL3 in combination with HLA-C1 has been reproducibly correlated with HCV infection outcome however, expression of an inhibitory KIR with HCV infection outcome was surprising at first. KIR2DL3 inhibits NK cells and NK cell activation is crucial for killing of virus infected hepatocytes either directly or by mediating the activation of the adaptive immune system (Golden-Mason et al. 2010, Pelletier et al. 2010). The described binding affinity of KIR2DL3 to HLA C1 was lower than for KIR2DL2 (Winter et al. 1998), thus HLA-C1 mediated inhibition of NK cells is lower in KIR2DL3 homozygous individuals. In HCV infection it was suggested that a weaker inhibitory control of NK cells might be beneficial as NK cells are more easily activated and are therefore able to contribute to resolution during acute infection or even mediate early immune control prior to seroconversion (Khakoo et al. 2004). This reduced threshold to activate NK cells could explain our observed increased cytokine production as well as degranulation ability in the low affinity ligand HLA-Bw4 80(T) carriers.

NK cells gain their functional competence during NK cell development in a process called NK cell education and NK cell education might provide an explanation for our observed superior NK cell functionality in the presence of multiple HLA-Bw4 alleles. Inhibitory receptor expression is essential to obtain functional cells that respond efficiently to a lack of self-MHC on the target cell (Kim et al. 2005). The number of inhibitory receptors as well as the presence of the ligand have a major impact on the functionality of NK cells (Sim et al. 2016). While homozygosity for the ligand for KIR2DL3 and KIR2DL1 expressing NK cells (C1/C1 or C2/C2) did not confer better education compared to heterozygosity of the ligand, KIR3DL1$^+$ NK cell function is dependent on the number of Bw4 motifs. The presence of two Bw4 copies was associated with a trend towards increased functionality of KIR3DL1$^+$ NK cells, thus multiple Bw4 copies resulted in enhanced education of KIR3DL1$^+$ NK cells (Sim et al. 2016). Comparable results were obtained in a study by Kim et al. (2008) demonstrating that KIR3DL1$^+$ NK cells from healthy individuals with the HLA-Bw4/Bw4 genotype had a higher ability to produce IFNγ compared to Bw4 heterozygous individuals and individuals lacking a Bw4 motif (Kim et al. 2008). Our results of healthy individuals were in line with these studies and confirmed a positive correlation between functionality of KIR3DL1$^+$ NK cells and Bw4 copy number. In addition, we could extend the observed superior functionality of NK cells in the presence of multiple Bw4 copies to increased TNFα production as well as enhanced degranulation ability. Furthermore, Boudreau et al. (2016) described that besides Bw4 copy number, functionality of KIR3DL1 NK cells was affected by the binding strength of KIR3DL1 to its ligand Bw4 as well as the receptor and ligand density on the cell surface. We observed congruent results for KIR3DL1 receptor density analysis whereas ligand density was not addressed in our study. Even though the differences were not statistically significant a tendency towards increased functionality of KIR3DL1$^+$ NK cells with multiple Bw4 copy numbers was observed in the PWID cohort as well.

Our study was the first to report that KIR3DL1 in combination with HLA-Bw4 was associated with resolution of HCV infection and being the only study so far that demonstrated a beneficial effect of HLA-Bw4 80(T) for viral clearance. In line with our results, Ruiz-Extremera et al. (2017) recently described an influence of KIR3DL1 as

well as HLA-Bw4 on the natural course of HCV infection in a study analysing the Mother-to-child transmission of HCV. The study indicated, that homozygosity for KIR3DL1 and the presence of multiple HLA-Bw4 alleles in the mother were two independent predictors for clearance of HCV infection in the child. However, no correlation of the combination of both with infection outcome or transmission rate was described. In addition, the combination of KIR3DL1 and HLA-Bw4 alleles has recently been described to influence treatment outcome. Nozawa et al. (2013) and Umemura et al. (2014) demonstrated that the KIR3DL1/HLA-Bw4 genotype was an independent predictor for SVR in a genotype 1b infected cohort from Japan after combination therapy with either PEGylated IFNα and ribavirin or after treatment with a triple therapy of telaprevir, PEGylated IFNα and ribavirin.

Furthermore, KIR3DL1 and Bw4 as well as the combination of both have been associated with beneficial effects in other diseases. In cancer, the genetic constellation of KIR3DL1 and HLA-Bw4 has been linked to the protection from diffuse large B-cell lymphoma and significant better overall survival in metastatic colorectal cancer patients (Karabon et al. 2011, De Re et al. 2014, Vejbaesya et al. 2014). Patients with neuroblastoma or Non-Hodgkin's Lymphoma harbouring the KIR3DL1/HLA-Bw4 genotype showed improved clinical outcome, especially individuals carrying the Bw4 80(T) motif. They reported that for monoclonal antibody based immunotherapy success, a weaker NK cell inhibition mediated by HLA-Bw4 80(T) was beneficial (Erbe et al. 2017), thus proposing the same mechanism for advantageous outcome as our HCV study. Moreover, the low-affinity ligand HLA-Bw4 80(T) has been associated with reduced relapse rates in acute myeloid leukaemia (AML) patients and multiple copies of HLA-Bw4 80(T) further decreased the incidence of AML relapses (Marra et al. 2015).

Furthermore, the HLA-Bw4 motif was correlated with a significant advantage in HIV infection outcome, as homozygosity for HLA-Bw4 was associated with suppression of HIV viremia, delayed HIV progression and thus delayed acquired immunodeficiency syndrome (AIDS) pathogenesis (Flores-Villanueva et al. 2001). Interestingly, the intracellularly retained KIR3DL1*004 in the presence of HLA-Bw4 was protective in HIV infection and influenced AIDS progression as well as plasma HIV RNA level (Martin et al. 2007). In contrast to our study, in HIV infection KIR3DL1 combined with HLA-Bw4 80(I) rather than HLA-Bw4 80(T) has been correlated with a significantly slower progression from HIV infection to AIDS (Martin et al. 2007) which indicates

that the impact of different combinations of KIR3DL1 alleles and their ligands varies between HIV and HCV infection. Numerous studies linked the Bw4 80(I) motif harbouring HLA-B*57 allele to favourable HIV outcome. The genetic combination of KIR3DL1, especially high expressing alleles, and HLA-B*57 were associated with a reduced risk of HIV infection in exposed uninfected individuals and slower disease progression (Martin et al. 2007, Boulet et al. 2008). In addition, NK cells from individuals with this protective genotype showed enhanced functionality (Boulet et al. 2010). Since the observed increased functionality of KIR3DL1$^+$ NK cell in the context of Bw4 80(T) was only seen in the PWID cohort, further analysis of possible effects of protective alleles in HCV infection like HLA-B*27 adding to NK cell functionality was limited in our study. Out of the 90 analysed PWIDs only four expressed KIR3DL1 and carried a HLA-B*27 allele including two individuals with multiple Bw4 copies, thus not allowing a valid and reliable analysis of the impact of HLA-B*27 on NK cell function.

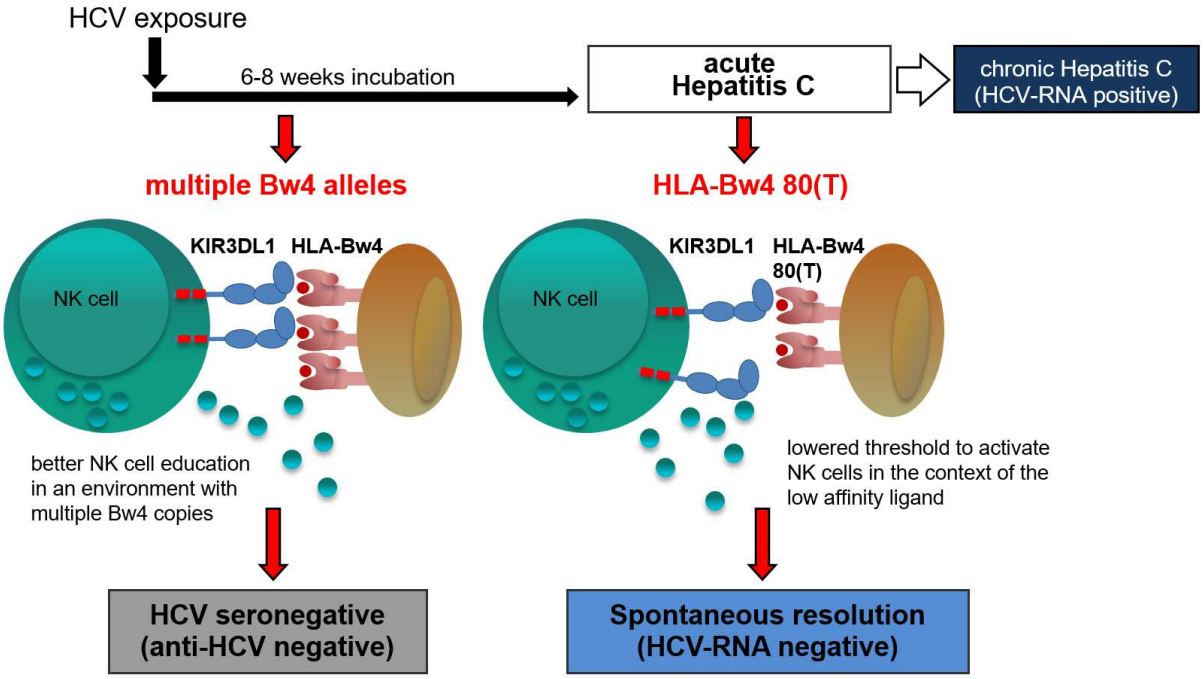

Figure 5.2: Graphical summary: association of KIR3DL1/HLA-Bw4 with HCV infection outcome and NK cell functionality in PWID

Upon HCV exposure, the majority of PWID develop chronic HCV infection. A second subgroup is able to clear HCV infection prior to seroconversion, thus remain HCV seronegative whereas a third group spontaneously resolves HCV infection after seroconversion. PWID with multiple Bw4 alleles are more likely to clear HCV infection before seroconversion compared to PWID with one or no Bw4 allele. In addition, increasing HLA-Bw4 copy numbers were correlated with a stepwise increase of functionality of KIR3DL1$^+$ NK cells in healthy individuals. Better NK cell functionality is probably mediated by enhanced education in the presence of multiple Bw4 copies. KIR3DL1 in combination with HLA-Bw4 80(T) was associated with spontaneous resolution of HCV infection after seroconversion and KIR3DL1$^+$ NK cells from HLA-Bw4 80(T)-positive PWID showed superior functionality compared to HLA-Bw4 80(I) positive PWID. Presumably a low activation threshold of NK cells in the presence of the low affinity ligand HLA-Bw4 80(T) results in enhanced functionality.

In summary, these data indicate that KIR3DL1 in the presence of HLA-Bw4 80(T) promotes spontaneous resolution of HCV during acute infection in a high-risk group of PWID, whereas this genetic combination however, does not mediate protection from HCV infection. In addition, multiple Bw4 copies were associated with HCV clearance prior to seroconversion, thus proposing a mechanism that mediates protection from HCV infection. Both genetic constellations were associated with superior NK cell functionality. Whereas enhanced KIR3DL1^{+} NK cell functionality in the context of multiple Bw4 copies is probably mediated by better NK cell education, the HLA-Bw4 80(T) effect in HCV infected individuals is most likely due to a lowered threshold to activate NK cells in the context of the low affinity ligand. Taken together this suggest that a superior NK cell functionality plays a critical role for HCV infection outcome in PWID.

5.3 DNA methylation of the *IFNG* locus

Our study showed that increased IFNγ production by KIR3DL1$^+$ NK cells in the context of multiple HLA-Bw4 copies was not epigenetically imprinted in the *IFNG* locus. DNA methylation of the *CNS1* region in KIR3DL1$^-$ and KIR3DL1$^+$ NK cells was independent of the Bw4 copy number. Interestingly, DNA methylation within the *IFNG* locus was significantly reduced in CMV$^+$ individuals. Moreover, hypomethylation of the *CNS1* region was correlated with the NKG2C expression on NK cells.

Previous studies reported a closed conformation and a highly methylated *CNS1* region as well as *IFNG* promoter in naïve T cells, whereas the *CNS1* region in TH1 cells was hypomethylated (Dong et al. 2013, Luetke-Eversloh et al. 2014). Moreover, the *IFNG* promoter was broadly demethylated in resting NK cells and methylation patterns were independent of NK cell activation (Wiencke et al. 2016). In accordance with these studies we observed similar methylation patterns of the *IFNG* locus comprising the *CNS1* region as well as the *IFNG* promoter, for naïve CD4$^+$ T cells, TH1 cells and NK cells. Furthermore, it has been described that the occurrence of hypomethylation of an upstream region regulating the transcription of IFNγ as part of the *IFNG* locus was associated with NK cell activation (Luetke-Eversloh et al. 2014). Moreover, demethylation of the *CNS1* region confered an increased IFNγ competence of NK cells (Luetke-Eversloh et al. 2014). However, our observed superior IFNγ production of KIR3DL1$^+$ NK cells could not be associated to hypomethylation occurring within the analysed *CNS1* region. Even though investigated epigenetic modifications did not provide an explanation for superior IFNγ functionality of NK cells from multiple HLA-Bw4 motif carriers, other epigenetic modification might be responsible for the observed differences in functionality between individuals lacking a Bw4 motif in comparison to Bw4$^+$ individuals. In addition to an increased IFNγ production, KIR3DL1$^+$ NK cells from individuals with multiple HLA-Bw4 motifs exhibited an enhanced ability to produce TNFα. DNA methylation might be the underlying mechanism mediating this functional difference, since previous reports described that the methylation pattern of the *TNFA* promoter correlated with gene transcription and demethylation of the *TNFA* promoter was described in activated NK cells (Wiencke et al. 2016).

In HCMV infection NKG2C^{high} NK cells show characteristics of adaptive immune cells, since they are clonally expanded after reactivation, highly functional and

long-lived. As these NK cells display features usually associated with adaptive memory cells they are termed memory NK cells (Sun et al. 2009). The expansion of this NKG2C^{high} memory NK cell population in HCMV infected individuals was associated with demethylation of the *CNS1* region. Moreover, the open configuration and accessibility of *CNS1* correlated with enhanced IFNγ competence (Luetke-Eversloh et al. 2014). Our study demonstrated an association between the occurring hypomethylation of the *CNS1* region and the CMV seropositivity of investigated individuals. In addition, we provided evidence that the epigenetic remodelling at this site within the *IFNG* locus was attributed to memory NK cells since we observed an inverse correlation between the DNA methylation and the frequency of NKG2C^{+} NK cells. Therefore, our study confirmed the previously reported association between NKG2C expression of CMV^{+} individuals and hypomethylation of the CpG sites within the *CNS1 region*. Collectively the existing data suggest that memory features of NK cells are epigenetically regulated.

In summary, our data confirms that NK cell memory features, which are attributed to NKG2C expressing NK cells in CMV positive individuals, are epigenetically imprinted within the *CNS1* region of the *IFNG* locus, whereas the significantly enhanced IFNγ capacity of KIR3DL1^{+} NK in the context of multiple HLA-Bw4 motifs is not epigenetically regulated by DNA methylation.

5.4 Future directions

5.4.1 iNKT cells in HCV infection

The impact of the activated iNKT cell status in HCV-RNA positive PWID on their functionality is not fully understood. It is reported that iNKT cell activation leads to an upregulation of FasL (Nakagawa et al. 2001), indicating that iNKT cells might directly induce liver injury through the Fas-FasL signalling pathway. This effector mechanisms could differ between individuals with different infection outcome, thus might provide functional explanations for the observed activated phenotype of iNKT cells in HCV infection. Therefore, further analysis of iNKT cell functions are necessary to understand the impact of iNKT cells in HCV infection outcome in depth. In addition, the role of iNKT cells in the activation of innate as well as adaptive immune cells should be further addressed in the context of HCV infection. The absence of NKT cells in HBV infection reduced HBV specific T cell responses (Zeissig et al. 2012). In addition, IFNγ produced by activated iNKT cells in mice increased $CD8^+$ T cell responses in a hepatic tumour mouse model (Nakagawa et al. 2001) which leads to the assumption that iNKT cell activation induced by αGalCer might positively affect $CD8^+$ T cell responses in HCV infection. Furthermore, direct antiviral effects of iNKT cells could be determined in cell culture based replicon systems. Recently, a mouse model of an HCV-related hepacivirus, that displays similarities with HCV infections in humans, was established (Billerbeck et al. 2017). Therefore, this new mouse model represents a suitable method to elaborately study the role of iNKT and non-invariant NKT cells in acute and chronic HCV infection.

5.4.2 NK cells in HCV infection

Here, we reported that the combination of KIR3DL1 and HLA-Bw4 80(T) was associated with spontaneous immune control of HCV infection in a high-risk cohort of PWID. Moreover, HLA-B*27 was reproducibly linked to spontaneous resolution of HCV infection and escape mutations within the presented epitope were associated with viral persistence. Interestingly, KIR3DL1 is not only able to bind to the Bw4 motif from HLA-A and HLA-B molecules but also makes contact to the presented peptide (reviewed in Parham et al. 2012, and O'Connor et al. 2013). Thus, it might be of interest to determine if viral peptide variants presented by a HLA-B*27 allele that harbours a Bw4 80(T) motif like HLA-B*27:05 modulate NK cell responses. Moreover, future analysis could determine if mutations within the presented viral epitope which confer T cell escape can also escape NK cell recognition.

6 Appendix

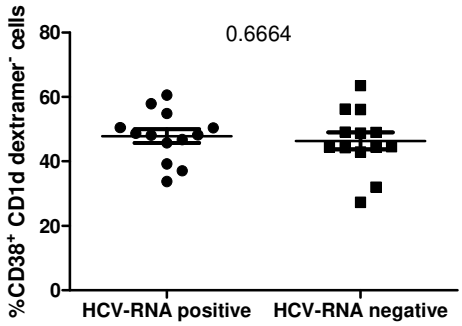

Figure 6.1: Expression of CD38 on CD1d dextramer negative lymphocytes

CD38 expression was analysed on CD1d dextramer negative lymphocytes from 13 HCV-RNA positive and 13 HCV-RNA negative individuals. The frequency of $CD38^+$ non iNKT cells is depicted and an unpaired t-test was used to determine significance. The mean ± SEM is shown.

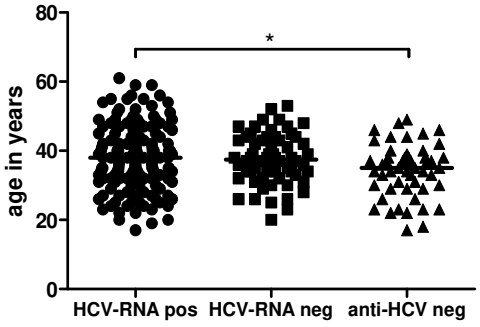

Figure 6.2: Age distribution within the PWID cohort

The age in years is depicted for every individual PWID that was included in the genetic KIR/KIR ligand association study. The median age of HCV-RNA positive (n=151), HCV-RNA negative (n=64) and anti-HCV negative (n=51) PWID is indicated. Significance was calculated by one-way ANOVA ($p \leq 0.05$).

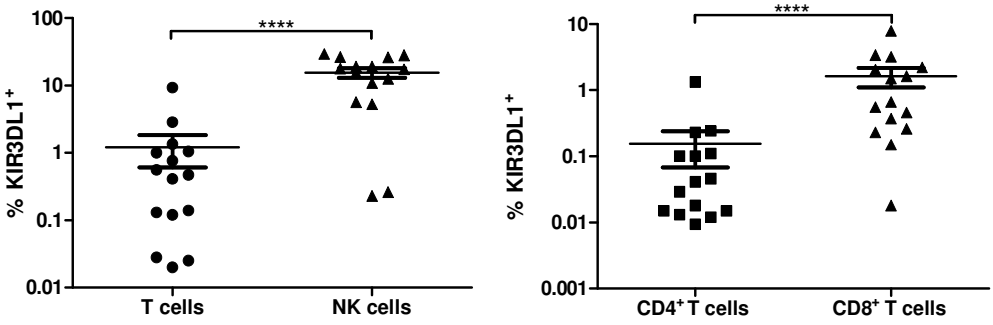

Figure 6.3: Frequency of KIR3DL1 expressing T cells

The frequency of $KIR3DL1^+$ T cells was determined in PBMCs from 15 healthy individuals. $KIR3DL1^+$ T cells were defined as $CD3^+$ single, viable lymphocytes and were compared to KIR3DL1 expressing NK cells. $CD3^+$ T cells were further subdivided into $CD4^+$ and $CD8^+$ cells. Significance was calculated by the use of the Wilcoxon signed rank test (****$p \leq 0.0001$) and the mean ± SEM is depicted.

7 Summary

Worldwide 71 million people are chronically infected with HCV and each year approximately 400 000 people die from HCV associated liver diseases. The most important risk factor in western industrialised countries to acquire HCV infection is intravenous drug use. For this reason, we utilised a cohort of people who inject drugs (PWID) to comparatively analyse PWID developing chronic infection (HCV-RNA positive), spontaneously resolving HCV infection (HCV-RNA negative) and PWID remaining anti HCV seronegative (anti-HCV negative), to provide novel insights into the contribution of innate and adaptive immune mechanisms to HCV infection outcome.

The role of Natural Killer T (NKT) cells, which share characteristics of innate and adaptive immune cells, for the outcome of HCV infection is poorly defined. Invariant NKT (iNKT) cells, a subset of NKT cells, recognise glycolipid antigens such as α galactosylceramide (αGalCer) presented by the non-classical MHC molecule CD1d. Decreased iNKT cell frequencies have been reported in chronically HCV infected patients, however, contradicting reports exist. In this study iNKT cell frequencies did not differ between HCV-RNA positive (n=28) and HCV-RNA negative (n=33) PWID. Interestingly, phenotypic analysis of iNKT cells of chronically infected PWID showed significantly higher expression of the activation marker CD38, however, this was not associated with apparent differences in PMA/ionomycin-stimulated effector functions. In addition, treatment with a combination of IL12, -15 and -18, which is described to activate NK cells, led to a significant increase in $CD38^+$ iNKT cells. Since IFN stimulated genes are induced in patients with chronic infection the influence of IFNs on iNKT cell activation was addressed as well. Although no direct effect of IFNα on iNKT cell activation was observed, CD1d was upregulated on peripheral monocytes, the cell type with the highest expression of CD1d in the periphery. However, CD1d levels on monocytes, did not differ between PWID with chronic and resolved HCV infection. Nevertheless, presence of monocytes promoted iNKT cell expansion and activation, as depletion of monocytes significantly reduced the frequency of $CD38^+$ iNKT cells. Furthermore, CD1d transcript levels were significantly higher in HCV-infected liver compared to HBV-infected liver. Collectively the data demonstrate that iNKT cells are activated in chronically HCV infected PWID, possibly via type I interferon-induced upregulation of CD1d on monocytes or other liver resident cells or via direct activation by

pro-inflammatory cytokines. However, the functional consequences of increased activation of iNKT cells are not fully defined.

While iNKT cells are still poorly covered by scientific reports in HCV infection there is growing evidence for the importance of NK cells for HCV infection outcome. Genetic association studies revealed that specific genetically determined combinations of NK cell-receptors and their ligands are associated with differential outcome of HCV infection. NK cell function is regulated by a set of inhibitory and activating receptors including killer-cell immunoglobulin-like receptors (KIRs). A previous study in our lab identified that KIR3DL1/HLA-Bw4 80(T) was associated with spontaneous clearance of HCV infection in our PWID cohort (n=266) which was validated in a second PWID cohort from North America (n=342). For this reason, this study focused on the functional characterisation of NK cells according to the identified KIR/KIR-ligand genotype. Besides PWID, healthy individuals (n=120) were included in the analysis as well. KIR3DL1$^+$ NK cells from HLA-Bw4 80(T) positive PWID showed superior functionality compared to HLA-Bw4 80(I) positive PWID. However, this differential impact was not observed in healthy donors. Moreover, the frequency of individuals with multiple HLA-Bw4 alleles was significantly higher in HCV-RNA negative as well as anti-HCV negative PWID compared to HCV-RNA positive PWID and HLA-Bw4 copy number strongly correlated with the functionality of KIR3DL1$^+$ NK cells in healthy individuals. HLA-Bw4 80(T) and multiple HLA-Bw4 copies in combination with KIR3DL1 are associated with protection against chronic HCV in PWID by distinct mechanisms. Better education of KIR3DL1$^+$ NK cells in the presence of multiple HLA-Bw4 copies is beneficial prior to seroconversion whereas HLA-Bw4 80(T) may be beneficial during acute hepatitis C. To determine possible underlying mechanisms leading to the superior functionality of KIR3DL1$^+$ NK cells, the DNA methylation pattern of the *IFNG* locus was analysed, since demethylation of the *IFNG* locus is generally associated with increased *IFNG* transcription. Unfortunately, the significantly enhanced IFNγ capacity of KIR3DL1$^+$ NK cells from multiple HLA-Bw4 motif carriers was not epigenetically regulated by DNA methylation. However, our data could confirm that NK cell memory features, attributed to NKG2C expressing NK cells in CMV positive individuals, are epigenetically imprinted within the *IFNG* locus.

In summary, these studies contribute novel evidence for the importance of NK cells and iNKT cells for HCV infection outcome and describe possible underlying mechanisms. However, further research is needed to elucidate for instance, the role of HCV peptides presented by HLA alleles harbouring a Bw4 motif on the function of KIR3DL1$^+$ NK cells or the exact mechanism leading to the activated state of iNKT cells during chronic HCV infection.

8 Zusammenfassung

Nach Schätzung der WHO sind zurzeit 71 Millionen Menschen weltweit chronisch mit dem Hepatis C Virus (HCV) infiziert und jährlich sterben etwa 400.000 Menschen an den Folgen einer HCV induzierten Leberkrankungen. In westlichen Industrieländern stellt der intravenöse Drogenkonsum hierbei den höchsten Risikofaktor dar, sich mit HCV zu infizieren. Um neue Erkenntnisse über die Rolle des natürlichen und angeboren Immunsystems in der HCV Infektion zu gewinnen, wurde einer Kohorte von intravenös Drogen gebrauchenden Menschen („people who inject drugs“ PWID) analysiert. Hierbei wurden PWID mit chronischer HCV-Infektion (HCV-RNA positiv), ausgeheilter HCV-Infektion (HCV-RNA negativ) und HCV seronegative PWID (anti-HCV negativ) miteinander verglichen.

Welche Rolle Natürliche Killer T (NKT) Zellen, die sowohl Eigenschaften von innaten als auch adaptiven Immunzellen aufweisen, bei dem Verlauf einer HCV Infektion spielen ist nicht klar definiert. Invariante NKT (iNKT) Zellen stellen eine Subgruppe der NKT Zellen dar und erkennen Glycolipid Antigene wie z.B. α galactosylceramid (αGalCer), die durch das nicht-klassische MHC Molekül CD1d präsentiert werden. Die Untersuchung der iNKT Zellfrequenzen zeigte keine Unterschiede zwischen HCV-RNA positiven (n=28) und HCV-RNA negativen (n=33) PWID. Interessanterweise ergab die phänotypische Analyse, dass sich iNKT Zellen von chronisch HCV infizierten PWID durch eine signifikant erhöhte Expression des Aktivierungsmarkers CD38 auszeichnen. Allerdings konnten keine funktionellen Unterschiede mit diesem aktivierten Phänotyp assoziiert werden. Zusätzlich wurde die Auswirkung von Interferonen auf iNKT Zellen in dieser Studie adressiert. Während kein direkter Effekt von IFNα oder IFNλ festgestellt werden konnte, führte eine Kombination von NK Zell aktivierenden Zytokinen (IL12, IL15 und IL18) zu einer signifikanten Erhöhung der $CD38^+$ iNKT Zellen. Auch wenn gezeigt werden konnte, dass der Ligand von iNKT Zellen durch IFNα Stimulation hochreguliert wird, konnte kein Unterschied in der CD1d Expression auf peripheren Monozyten, welche in der Peripherie den Zelltyp mit der höchsten CD1d Expression darstellen, zwischen HCV-RNA positiven und HCV-RNA negativen PWID festgestellt werden. Dennoch nehmen Monozyten eine wichtige Rolle in der Expansion und Aktivierung von iNKT Zellen ein, da eine Depletierung selbiger eine signifikant verminderte Frequenz von $CD38^+$ iNKT Zellen zur Folge hat. Des Weiteren ergaben Leberanalysen, dass HCV-infizierte Lebern im Vergleich zu HBV-infizierten Lebern eine signifikant erhöhte CD1d Transkription aufweisen. Zusammengefasst weisen unsere Daten darauf hin,

dass iNKT Zellen in der chronischen HCV Infektion aktiviert sind. Sowohl eine Typ I Interferon induzierte Hochregulation von CD1d auf Monozyten oder anderen Zellen in der Leber als auch das proinflammatorische Zytokine Milieu sind potentiell für die Modulation des Aktivierungsstatus von iNKT Zellen in der HCV Infektion verantwortlich.

Während es kaum wissenschaftliche Berichte gibt, die sich mit iNKT Zellen im Kontext der HCV Infektion auseinandersetzen, steigt die Zahl an Belegen, die NK Zellen eine wichtige Bedeutung für die Prävention und Kontrolle einer HCV Infektion zusprechen. Verschiedene Assoziationsstudien konnten bereits zeigen, dass genetisch determinierten Kombinationen von NK Zell Rezeptoren und ihren Liganden mit der HCV Verlaufsprognose assoziiert sind. Die NK Zell Funktion wird durch aktivierende und inhibierende Rezeptoren einschließlich der Killerzell-Immunglobulin-ähnlichen Rezeptoren (KIR) reguliert. In einer vorangegangenen Studie wurde mit Hilfe einer multivariaten logistischen Regressionsanalyse KIR3DL1/HLA-Bw4 80(T) mit der spontanen Ausheilung einer HCV Infektion in unserer PWID Kohorte (n=266) assoziiert. Diese Assoziation konnte in einer zweiten anti-HCV positiven PWID Kohorte (n=342) aus Nordamerika validiert werden. Auf Grund dessen wurden in der vorliegenden Studie NK Zellen anhand ihres KIR/KIR-Liganden Genotyps funktionell charakterisiert. Neben PWID wurden auch gesunde Proben (n=120) in der Analyse inkludiert. Es konnte gezeigt werden, dass KIR3DL1$^+$ NK Zellen von HLA-Bw4 80(T) positiven PWID verglichen mit HLA-Bw4 80(I) positiven PWID eine signifikant erhöhte NK Zell Funktionalität aufweisen. Hingegen konnte kein funktioneller Einfluss dieses Genotyps in gesunden Individuen beobachtet werden. Darüber hinaus wurde gezeigt, dass multiple HLA-Bw4 Allele, deren Frequenz signifikant in HCV-RNA negativen und anti-HCV negativen PWID im Vergleich zu HCV-RNA positiven PWID erhöht ist, auch mit einer starken NK Zell Funktionalität einhergehen. Zusammenfassend können sowohl HLA-Bw4 80(T) als auch multiple Kopien von HLA-Bw4 in Kombination mit KIR3DL1 mit der Protektion gegen eine chronische Hepatitis C assoziiert werden. Diese wird über verschiedene Mechanismen vermittelt: während eine bessere NK Zell Lizensierung durch die Anwesenheit von multiplen HLA-Bw4 Kopien vor der Serokonversion vorteilhaft ist, könnte HLA-Bw4 80(T) während eine akuten HCV Infektion von Vorteil sein. Epigenetische Veränderung könnten eine mögliche Erklärung für die erhöhte IFNγ Produktion von KIR3DL1$^+$ NK Zellen im Kontext von multiplen HLA-Bw4 Allelen bieten. Hierfür wurde die DNA Demethylierung des *IFNG*

Lokus, welche generell mit einer erhöhten Transkription assoziiert ist, analysiert. Es konnte jedoch kein Zusammenhang zwischen dem Methylierungsstatus und der Anzahl an HLA-Bw4 Kopien hergestellt werden. Hingegen konnten vorausgegangene Studien bestätigt werden, die besagen, dass Memory-Eigenschaften von NKG2C exprimierenden NK Zellen in CMV positiven Individuen epigenetisch reguliert sind.

Abschließend lässt sich feststellen, dass die hier präsentierten Studien neue Beweise für die Bedeutsamkeit von NK und iNKT Zellen für den HCV Infektionsverlauf liefern und Hinweise auf mögliche zugrundeliegende Mechanismen geben. Dennoch sind weitere Arbeiten notwendig die z.B. klären ob HCV Peptide, die durch Bw4 Motiv tragende HLA Allele präsentiert werden, einen Einfluss auf die Funktion von KIR3DL1^{+} NK Zellen haben oder welche Mechanismen genau während der chronischen Infektion zu einer Aktivierung von iNKT Zellen führen.

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10 List of abbreviations

ADCC	antibody dependent cell cytotoxicity
αGalCer	α-Galactosylceramide
AIDS	acquired immunodeficiency syndrome
AML	acute myeloid leukaemia
APC	allophycocyanin
BD	Beckton Dickinson
BEC	Beckman Coulter
BFA	brefeldin A
BV	Brilliant Violet
CD	cluster of differentiation
CI	confidence interval
CNS	conserved noncoding sequence
Cy	cyanin
DAAs	direct-acting antivirals
DC	dendritic cell
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide Triphosphate
DP	double positive
eBio	eBioscience
et al.	et alii (and others)
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	hepatocellular carcinoma
HCMV	human cytomegalovirus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	human leukocyte antigen
HSV	herpes simplex virus
IFN	interferon
IFNα	interferon alpha
IFNγ	interferon gamma
IFNλ	interferon lambda
Ig	immunoglobulin
iGb3	isoglobotrihexosylceramide
IHL	intrahepatic lymphocytes
IL	interleukin
iNKT	invariant NKT
ISGs	interferon-stimulated genes
ITAM	immunoreceptor tyrosine-based activation motif

ITIM	immunoreceptor tyrosine-based inhibition motif
IU	international units
kb	kilo bases
KIR	killer cell immunoglobulin-like receptor
MAIT	mucosal associated invariant T
5mC	five-methyl-cytosine
MDA 5	melanoma differentiation associated protein 5
MHC	major histocompatibility complex
mRNA	messenger RNA
MSM	men having sex with men
n.a.	not applicable
NANBH	non-A non-B hepatitis
NCR	natural cytotoxicity receptor
n.d.	not determined
NK	Natural Killer
NKT	Natural Killer T
OR	odds ratio
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate-Buffered Saline
PCR	polymerase chain reaction
PD1	programmed death 1
PE	phycoerythrin
PEG	polyethylene glycol
PerCP	peridinin chlorophyll
PLZF	promyelocytic leukaemia zinc finger protein
PMA	phorbol myristate acetate
PWID	people who inject drugs
RAS	resistance associated substitutions
RIG I	retinoic acid inducible gene I
RNA	ribonucleic acid
RT	room temperature
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SVR	sustained virological response
TCR	T cell receptor
TH	T helper
TLR3	Toll like receptor 3
TNFα	tumour necrosis factor alpha
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
Treg	regulatory T cell
TSS	transcriptional start site
UTR	untranslated region
WHO	World Health Organization

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13 Publications

- **Senff T**, Thöns C, Peters M, Scherbaum N and Timm J. Comparative analysis of CD1d-restricted natural killer T cells in people who inject drugs with chronic or spontaneously resolved hepatitis C. (2017) **in preparation**
- Thöns C, **Senff T**, Bäcker E, Zimmermann A, Uhrberg M, Lang P and Timm J. Cytomegalovirus-associated NKG2Cpos NK cells inhibit virus-specific CD8 T cells. JCI (2017) **submitted**
- Thöns C**#**, **Senff T#**, Hydes TJ, Manser AR, Heinemann FM, Heinold A, Heilmann M, Kim AY, Uhrberg M, Scherbaum N, Lauer GM, Khakoo SI and Timm J. HLA-Bw4 80(T) and multiple HLA-Bw4 copies combined with KIR3DL1 associate with spontaneous clearance of HCV infection in people who inject drugs. J Hepatol (2017) **# equal contribution**

14 Contributions at conferences

- **T. Senff**, C. Thöns, M. Peters, N. Scherbaum, J. Timm; Comparative analysis of CD1d-restricted natural killer T cells in people who inject drugs with chronic or spontaneously resolved hepatitis C; Natural Killer Cell Symposium 2017, 09-11.03.2017 Düsseldorf; poster presentation
- **T. Senff*,** C. Thöns*, T. Hydes, A. R. Manser, F. M. Heinemann, A. Heinold, M. Heilmann, A. Y. Kim, M. Uhrberg, N. Scherbaum, G. M. Lauer, S. I. Khakoo, J. Timm; HLA-Bw4 80(T) and multiple HLA-Bw4 copies combined with KIR3DL1 are associated with superior immune control of HCV infection in people who inject drugs; Natural Killer Cell Symposium 2017; 09-11.03.2017 Düsseldorf; poster presentation
- **T. Senff*,** C. Thöns*, T. Hydes, F. Heinemann, A. Heinold, M.Heilmann, M. Uhrberg, N. Scherbaum, S. Khakoo, J. Timm; KIR3DL1 in combination with HLA-Bw4-80T is associated with superior functionality of NK cells and spontaneous immune control of HCV infection in people who inject drugs; 46th Annual Meeting of the German Society for Immunology, 27.09.2016-30.09.2016 Hamburg; **oral presentation**
- **T. Senff**, C. Thöns, N. Scherbaum, J. Timm; Comparative analysis of CD1d-restricted natural killer T cells in chronic or spontaneously resolved hepatitis C; 46th Annual Meeting of the German Society for Immunology, 27.09.2016-30.09.2016 Hamburg; poster presentation
- **T. Senff**, C. Thöns, N. Scherbaum, J. Timm; Comparative analysis of CD1d-restricted natural killer T cells in chronic or spontaneously resolved hepatitis C; 4th Translational DZIF School; 21–23 September 2016 Lübeck; poster walk
- **T. Senff*,** C. Thöns*, T. Hydes, F. Heinemann, A. Heinold, M.Heilmann, M. Uhrberg, N. Scherbaum, S. Khakoo, J. Timm; KIR3DL1 in combination with HLA-Bw4-80T is associated with superior functionality of NK cells and spontaneous immune control of HCV infection in people who inject drugs; EASL 2016; 13-17 April 2016; Barcelona Spain; poster walk
- **T. Senff*,** C. Thöns*, T. Hydes, F. Heinemann, A. Heinold, M.Heilmann, M. Uhrberg, N. Scherbaum, S. Khakoo, J. Timm ; KIR3DL1 in combination with HLA-Bw4-80T is associated with superior functionality of NK cells and spontaneous immune control of HCV infection in people who inject drugs; 6th Annual Meeting

of the Society for Virology; 06-09 April 2016; Münster Germany; poster presentation

- **T. Senff**, C. Thöns, N. Scherbaum, J. Timm; Comparative analysis of CD1 d-restricted natural killer T cells in people who inject drugs with chronic or spontaneously resolved hepatitis C; Natural Killer Cell Symposium 2015; 7-9.10.2015; Göttingen Germany; poster presentation and **one minute oral presentation**
- **T. Senff**, C. Thöns, N. Scherbaum, J. Timm; The role of CD1d-restricted natural killer T cells in HCV infection; 3. International Symposium "Viral Strategies of Immune Evasion" VISTRIE; 22.05.2015, Braunschweig Germany; poster presentation
- **T. Senff**, C.Thöns, N. Scherbaum, J. Timm; The role of CD1d-restricted natural killer T cells in HCV infection; 25th Annual Meeting of the Society for Virology; 18-21 March 2015; Bochum Germany; poster presentation
- **T. Senff**, C. Thöns, N. Scherbaum, J. Timm; Comparative analysis of CD1d-restricted natural killer T cells in people who inject drugs with chronic or spontaneously resolved hepatitis C; 32. Annual Meeting of the German Association for the Study of the Liver (GASL) in Düsseldorf; 22-23 January 2016; Düsseldorf Germany; Z Gastroenterol 2015; 53 - A5_8 DOI: 10.1055/s-0035-1568101, poster presentation
- **Presenting author; *equal contribution**

Further attended conference:

- UK NK Cell Meeting 2017; 6 January 2017; UCL London UK

15 Acknowledgements

Now is the time to say, “thank you”!

First of all, I would like to express all my gratitude to my supervisor and mentor Prof. Dr. Jörg Timm who provided me with the opportunity to work on several challenging and fascinating projects during my dissertation. Throughout all the years of my PhD, he guided and supported me in every way possible. Thanks to him I learnt a lot and he was enormously helpful for my scientific as well as personal development.

Furthermore, I would like to thank my second supervisor Prof. Dr. Astrid Westendorf for contributing valuable ideas and further directions for this project.

Additionally, I would like to thank all members of the Institute of Virology for making sure the lab was a great place to work in. My sincere thanks go to all the “AG Timm” members (past and present) for the support and the help in the lab as well as for the productive scientific discussions. Furthermore, I would like to thank Alex Graupner and Eugen Bäcker for their technical assistance. I also want to thank Christine Thöns, Christopher Menne, Eugen Bäcker, Janine Brinkmann, “Jonny” Walker, Tatjana Schwarz and Ole Heidkamp for all the laughter and the coffee chats! Thank you, Nadine Lübke and Andreas Walker, for always providing a full drawer of sweets that got me through some long days. Special thanks to Janine Brinkmann for always seeing the good in everything and everyone. Your support during this PhD time was exceptional and you are not only a valuable lab member but also became a very good friend! I am deeply grateful for all of you, that contributed to a wonderful atmosphere and all the fun I had during my PhD in the lab and the office!

I would also like to thank all the collaborators, especially Prof. Salim Khakoo for providing me with the opportunity to join his lab for three months. Thanks to all “NK group” and all “Level E” members who helped me around the lab during this time. I am especially grateful for Pauline Rettman, Berenice Mbiribindi and Leidy Bastidas Legarda who showed me around Southampton and always managed to brighten up the wet and grey UK weather!

Furthermore, I want to thank all my friends who took my mind off science and for motivating me to never stop trying.

Above all I would like to thank my soulmate and partner in crime Martin Schmitz for his company, love, patience, support and encouragement during all the time we known each other. Your always patient with me during challenging time periods and you know how to motivate me or calm me down when needed. You are extraordinary and always make me laugh. Thanks for always being there for me, I love you!

16 Curriculum vitae

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17 Declarations

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Hiermit erkläre ich, gem. § 7Abs. (2) d) + 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, bei der Abfassung der Dissertation nur die angegebenen Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Prof. Dr. Jörg Timm