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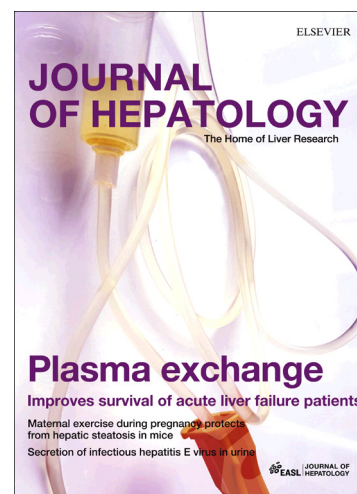
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HLA-Bw4 80(T) and multiple HLA-Bw4 copies combined with KIR3DL1 associate with spontaneous clearance of HCV infection in people who inject drugs

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	concept and design	sample collection	acquisition of data	analysis of data	statistical analysis	drafting of manuscript
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Martin Heilmann		X	X			
Arthur Y. Kim	X	X				X
Markus Uhrberg	X			X		X
Nobert Scherbaum	X	X	X			X
Georg M. Lauer	X	X				X
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Joerg Timm	X			X	X	X

Abstract:

Background and aims: NK cell function is regulated by inhibitory and activating receptors including killer-cell immunoglobulin-like receptors (KIRs). Here, we analyzed the impact of different KIR/KIR-ligand genotypes on the outcome of HCV infection in people who inject drugs (PWID). KIR/KIR-ligand genotypes associated with spontaneous clearance of HCV infection were identified in a cohort of PWID from Germany (n=266) and further validated in a second anti-HCV positive cohort of PWID recruited in North America (n=342). Moreover, NK cells of PWID and healthy donors were functionally characterized according to their KIR/KIR-ligand genotype by flow cytometry.

Results: Multivariate logistic regression analysis revealed that KIR3DL1/HLA-Bw4 80(T) was associated with spontaneous clearance of HCV infection in PWID, which was confirmed in the PWID cohort from North America. Moreover, compared with PWID with detectable HCV-RNA the frequency of individuals with multiple HLA-Bw4 alleles was significantly higher in anti-HCV positive PWID with resolved HCV infection (29.7% vs. 15.2%; p=0.0229) and in anti-HCV seronegative PWID (39.2%; p=0.0006). KIR3DL1⁺ NK cells from HLA-Bw4 80(T)-positive PWID showed superior functionality compared to HLA-Bw4 80(I)-positive PWID. This differential impact was not observed in healthy donors; however the HLA-Bw4 copy number strongly correlated with the functionality of KIR3DL1⁺ NK cells.

Conclusions: HLA-Bw4-80(T) and multiple HLA-Bw4 copies in combination with KIR3DL1 are associated with protection against chronic hepatitis C in PWID by distinct mechanisms. Better licensing 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.

Lay summary: NK cells are part of the innate immune system and are regulated by a complex network of activating and inhibiting receptors. The regulating receptor-ligand pairs of an individual are genetically determined. Here, we identified a particular set of ligand and receptor genes that associated with better functionality of NK cells and better outcome upon exposure to HCV in a high risk group.

INTRODUCTION

People who inject drugs (PWID) are the most important risk-group for new infections with the hepatitis C virus (HCV)(1). Sharing needles and other injection equipment is common practice and associated with high risk for HCV transmissions and incident infections resulting in prevalence rates of anti-HCV up to 80% in some PWID cohorts(1). Although in Germany strategies for prevention of HCV transmission among PWID have been implemented in large cities, about 65% of PWID are still anti-HCV positive as a marker for past exposure to HCV(2). Approximately 60-70% of the anti-HCV positive PWID are also positive for HCV-RNA as a marker for ongoing viral replication and are therefore at risk for onward transmission and progressive liver disease. In turn, the remaining 30-40% achieved immune control and spontaneously cleared viremia. Notably, epidemiological and immunological evidence suggest that repeated exposures to different HCV isolates are common in high-risk groups and individuals with multiple consecutive infections have been described(3-5). Moreover, there is growing evidence that at least a subgroup of anti-HCV seronegative PWID was also exposed to HCV based on the reported risk behavior and the observation that T cell responses against HCV are detectable (6, 7). This subgroup is characterized by both distinct immunogenetics and immune cell function compared to healthy non-exposed individuals (8-10), suggesting that early immune control prior to seroconversion is possible but not necessarily mediated by the same mechanisms that promote HCV clearance after seroconversion. On a cellular level, CD8⁺ T cells play a crucial role in HCV clearance(11). However, HCV-specific CD8⁺ T cells are only found in 25% to 58% of anti-HCV seronegative PWIDs (6, 7, 10) and become detectable only after weeks of infection (12). In contrast, NK cells are activated very early upon exposure (13) and potentially contribute to both protection from persistent infection prior to seroconversion and resolution of acute Hepatitis C (reviewed in (14)). Comparative analyses of individuals who spontaneously resolved HCV infection, individuals who develop chronic infection and HCV seronegative individuals with high-risk behavior provide an opportunity to understand the underlying mechanisms of protective immunity against HCV.

Numerous studies have highlighted substantial differences in adaptive immunity between patients with resolved HCV infection and patients with chronic infection (reviewed in(11)). Moreover, it has also been recognized that differences in innate immunity exist between both groups. A highly reproducible finding is the association between single nucleotide polymorphisms upstream of the *IFNL3* gene locus and spontaneous resolution of HCV infection (15-17). Genetic association studies also revealed that the genetically determined combinations of NK cell-receptors and its ligands are associated with differential outcome of HCV infection(18). Specifically, PWID who are homozygous for the killer cell immunoglobulin-like receptor (KIR) KIR2DL3 and the receptor-ligand HLA-C1 are significantly enriched in the group of patients with spontaneous immune control of HCV(19). This association was reproduced in different cohorts and was extended to anti-HCV seronegative high-risk PWID(8, 20-22). However, other genetic association studies did not confirm the same finding(23, 24) or revealed different associations between genetic KIR/KIR-ligand combinations and control of HCV(23, 25). Thus genetic determinants of immune control of HCV might differ between cohorts.

Here, the influence of the genetically determined combinations of KIR and KIR-ligands on the outcome of HCV infection was re-addressed in a single-center cohort of PWID collected in Germany. The KIR and HLA class I-genotype of a total of 266 well-defined PWID including 151 anti-HCV seropositive PWID with detectable HCV-RNA, 64 treatment-naïve anti-HCV seropositive PWID without detectable HCV-RNA and 51 anti-HCV seronegative PWID with high-risk behavior. On a genetic level HLA class I alleles with the Bw4 80(T) motif or multiple copies of HLA-Bw4 alleles in combination with its receptor KIR3DL1 were associated with a protective state against chronic HCV infection in PWID. This finding was confirmed in a cohort of 342 PWID (267 HCV-RNA positive and 75 HCV-RNA negative) recruited in North America. The relevance of the genetic association was supported by functional analyses of NK cells from PWID and from healthy donors. The results further corroborate an important role for NK cells in the outcome of

HCV infection in PWID and give insight into a possible and complex functional mechanism of the beneficial effect of KIR3DL1 in combination with its ligand HLA-Bw4.

ACCEPTED MANUSCRIPT

PATIENTS AND METHODS

Study subjects

Blood samples from patients with a history of injection drug use were collected from the ward for inpatient detoxification treatment of drug addicts or the clinic for opioid maintenance treatment (OMT) at the Department of Addictive Behavior and Addiction Medicine of the LVR-Hospital Essen, Hospital of the University of Duisburg-Essen. Written informed consent was obtained from all participants and the study was approved by the ethics committee of the Medical Faculty of the University of Duisburg-Essen. In this study, only samples from treatment-naïve patients were included. All samples were tested by CMIA for anti-HCV (Abbott) and by PCR for the presence of HCV-RNA by the Abbott RealTime HCV PCR assay with a detection limit of 12 IU/ml. PWID were classified in 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). For functional analyses of NK cells 30 representative patients of each group were selected. The characteristics of the complete PWID cohort and the patients selected for functional studies are shown in table 1. 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 (USA) with local Ethics Committee approval. PBMCs from healthy donors were processed from buffy coats obtained at the center for blood donation from the University Hospital Düsseldorf with local Ethics Committee approval. These samples were anonymized and no further donor information was available.

KIR and HLA genotyping

DNA of patients was extracted from PBMCs using spin columns (Qiagen, Hilden, Germany). The KIR genotyping was performed as previously described(10). HLA class I typing was performed by use of sequence-specific oligonucleotides (LABType methodology) both provided by One Lambda Inc. (Canoga

Park, CA). DNA-based KIR3DL1 allele subtyping and subgroup determination (null, low, high) was performed as previously described with adjusted cycling conditions (26).

Analysis of NK cells

PBMCs were stimulated in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES buffer, and 10 ng/ml Brefeldin A (Sigma Aldrich) with K562 at an effector-target ratio of 10:1 for 5 h. Subsequently NK cells were analyzed via flow cytometry. All antibodies used are indicated in the Supplementary CTAT Table (Table S3.). K562 cells were tested negative for mycoplasma.

Statistical analysis

Associations between KIR/KIR-ligands and infection were determined using univariate and multivariate analyses to determine factor selection and test independence. Multivariate logistic regression was applied using the backward stepwise procedure for all variables that were significant at univariate analysis. Relationships were estimated using the odds ratio (OR) and its 95% Confidence Intervals. Routine statistical software packages were used for the analyses (SPSS version 21, GraphPad Prism 6.0 software). A $p < 0.05$ was considered to be statistically significant. Statistical analyses of NK cell frequencies and function were performed using GraphPad Prism 5.04 software (GraphPad Software, San Diego California USA). 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. Three or more groups were compared by one-way analysis of variance (ANOVA) or a Kruskal-Wallis test.

RESULTS

The combination of KIR3DL1 with HLA-Bw4 80(T) and multiple HLA-Bw4 copies is associated with the outcome of HCV infection

To identify KIR/KIR-ligand combinations associated with spontaneous control or “resistance” to HCV infection in a high-risk group of PWID the KIR and HLA class I gene locus were typed in 151 HCV-RNA positive PWID (HCV-RNA positive), 64 untreated anti-HCV positive PWID without detectable HCV-RNA (HCV RNA negative) and 51 anti-HCV seronegative PWID (anti-HCV negative). Characteristics of all patients included in the genetic analysis are given in table 1. HLA class I typing was resolved at a 4-digit level to allow reliable discrimination between Bw4 80(I) and Bw4 80(T) encoding HLA-B genes and to identify Bw80(I) encoding HLA-A subtypes. How all observed HLA-A and -B alleles were grouped with respect to the HLA-Bw4 status is shown in table S1. In addition, the genotype of the single nucleotide polymorphisms rs12979860 (CC versus non-CC) adjacent to the interferon lambda gene locus was determined. Significant associations between KIR/KIR-ligand genotypes and HCV infection status are summarized in table 2. Consistent with previous reports (15-17) the rs12979860 CC-genotype was associated with an HCV-RNA negative status when HCV-RNA positive and HCV RNA negative anti-HCV seropositive PWID were compared ($p=0.005$). In an univariate analysis of all possible KIR/KIR-ligand combinations the genotypes KIR3DL1/HLA-Bw4 80(T) and to a weaker extent KIR2DL1/HLA-C2 were significantly enriched in seropositive HCV-RNA negative PWID compared to HCV-RNA positive PWID ($p=0.003$ and $p=0.014$). In a multivariate logistic regression analysis only the rs12979860 CC genotype and the combination of KIR3DL1 with HLA-Bw4 80(T) were confirmed to be associated with an HCV-RNA negative state in anti-HCV seropositive PWID (table 2). Notably, presence of HLA class I alleles carrying the Bw4 80(I)-motif in combination with KIR3DL1 was not associated with an HCV-RNA negative status in anti-HCV-seropositive PWID. None of the KIR/KIR-ligand combinations were associated with an anti-HCV seronegative state (table2).

Because the combination of KIR3DL1 and HLA-Bw4 80(T) has not previously been associated with HCV infection outcome we further validated the result in a separate cohort of anti-HCV seropositive PWID recruited in North America including 267 HCV-RNA positive and 75 HCV-RNA negative individuals. In this cohort individuals encoding the KIR3DL1/HLA-Bw4 80(T) genotype were also significantly enriched in the HCV-RNA negative group compared to the HCV-RNA positive group (44.0% vs. 28.8%; $p=0.017$) confirming the protective effect that was observed in the PWID cohort from Germany (Fig. 1). An overview of the HLA allele frequencies in both cohorts is given in Table S2.

It has been reported that the copy number of KIR-ligands can also influence the functionality of NK cells(27-29). We therefore addressed if the HLA-Bw4 copy number was associated with the HCV infection status in our PWID cohort. HCV-RNA positive PWID, HCV-RNA negative PWID and anti-HCV seronegative PWID were grouped according to the HLA-Bw4 copy number into PWID lacking a HLA-Bw4 allele, PWID carrying one HLA-Bw4 allele and PWID carrying two or more HLA-Bw4 alleles (table 3). PWID expressing two or more Bw4 motifs were strongly enriched in the anti-HCV negative group (39.2%; $p=0.0006$) compared to the HCV-RNA positive group (15.2%). The frequency of PWID with multiple Bw4 alleles was also higher in the anti-HCV positive HCV-RNA negative group (29.7%) compared to the HCV-RNA positive group ($p=0.0229$), however this finding was not confirmed in the PWID cohort from North America.

PWID with different HCV infection status have similar frequencies and function of KIR3DL1⁺ NK cells

To further address the impact of KIR3DL1⁺ NK cells in the context of HCV infection in PWID, peripheral blood mononuclear cells (PBMCs) from representative subgroups of the German cohort were analyzed by flow cytometry. The analysis was performed using 90 PWID possessing a KIR3DL1 gene including 30 HCV-RNA negative, 30 HCV-RNA positive and 30 anti-HCV seronegative PWID. KIR3DL1 expressing NK cells were identified using flow cytometry. For functional analysis PBMCs were stimulated with the HLA-devoid target cell line K562 and effector functions (IFN γ and TNF α secretion, CD107a expression) of

KIR3DL1⁺ NK cells were analyzed using flow cytometry. The gating strategy and representative dot plots are shown in Fig. S1. No differences in the frequencies of KIR3DL1⁺ NK cells (Fig. 2A) or KIR3DL1 MFI (data not shown) were observed when the different PWID groups were compared. Moreover, there were also no functional differences between KIR3DL1⁺ NK cells regarding secretion of IFN γ (Fig. 2B), TNF α or CD107a expression (data not shown) between the different groups.

As the KIR3DL1 gene locus is highly polymorphic and different alleles have been linked to different KIR3DL1 expression levels, a previously described multiplex PCR assay(26) was used to characterize the KIR3DL1 gene of 60 PWID included in the flow cytometric analysis in more detail. Based on the PCR results three groups were defined. PWID that were homozygous for non-expressing KIR3DL1 genes were grouped (KIR3DL1 null). PWID that were either homozygous for low expressing KIR3DL1 genes or encoded a KIR3DL1 low expressing gene in combination with either a non-expressing KIR3DL1 gene or a KIR3DS1 gene were grouped (KIR3DL1 low). PWID who were homozygous for high expressing KIR3DL1 genes or encoding one high expressing KIR3DL1 gene in combination with either a low or non-expressing KIR3DL1 gene or a KIR3DS1 gene were grouped (KIR3DL1 high). As previously described(30) the KIR3DL1 subgenotype closely correlated with the frequency of KIR3DL1⁺ NK cells (Fig. 2C) and KIR3DL1 expression level (data not shown). However, there was no difference between PWID with different HCV infection status.

HLA-Bw4 copy number correlates with NK cell functionality in healthy individuals but not in PWID

As the genetic analysis revealed that PWID with two or more HLA-Bw4 motifs were enriched in the HCV-RNA negative group and in the anti-HCV seronegative group, we next grouped PWID according to Bw4 copy number rather than HCV infection status and analyzed IFN γ , TNF α and CD107a expression in response to stimulation with K562 cells. Although there was a trend towards better functionality of NK cells from donors carrying multiple HLA-Bw4 motifs, we did not see significant differences between the

groups (Fig. 3 A-C). To validate the finding, we stimulated PBMCs isolated from 120 healthy donors using the same stimulation and staining protocol that was used to analyze the PWID cohort. In the healthy cohort a significant stepwise increase of IFN γ (Fig. 3D) and CD107a (Fig.3F) production was observed in individuals with no, one or at least two Bw4 motifs. In addition, healthy individuals with two or more Bw4 motifs produced significantly more TNF α than individuals with no or one Bw4 motif. These findings are in line with a previous study performed in healthy individuals(29).

HLA-Bw4 80(T) associates with better NK cell functionality in PWID but not in healthy controls

As the multivariate logistic regression analysis identified the combination of HLA-Bw4 80(T) and KIR3DL1 to be enriched in the HCV-RNA negative PWID group we next analyzed the influence of Bw4 80(T) on NK cell functionality. For that purpose we analyzed NK cells of PWID of all subgroups carrying a single HLA-Bw4 allele (Bw4 80(T) or Bw4 80(I)) to avoid effects mediated by the HLA-Bw4 copy number and included individuals lacking HLA-Bw4-alleles as a control group. In the same individual KIR3DL1⁺ NK cells produced significantly more IFN γ , TNF α and CD107a than KIR3DL1⁻ NK cells independent of the Bw4 ligand (Fig. S2). This observation was made in both healthy individuals and PWID (data not shown). Importantly, KIR3DL1⁺ NK cells of PWID encoding a Bw4 80(T) motif produced significantly more IFN γ , TNF α and CD107a compared to KIR3DL1⁺ NK cells of individuals encoding a Bw4 80(I) motif (IFN γ : p<0.05; TNF α : p<0.01; CD107a: p<0.05; Fig.4 A-C) and significantly more TNF α and CD107a than PWID with no Bw4 motif (TNF α : p<0.05; CD107a: p<0.05; Fig.4 A-C). Interestingly, this differential functionality of KIR3DL1⁺ NK cells in the presence of HLA-Bw4 80(T) and Bw4 80(I) was not observed in healthy donors (Fig. 4D-F).

DISCUSSION

In this study the influence of the genetically determined KIR/KIR-ligand combination on the outcome of HCV infection was analyzed in a high-risk cohort of HCV-treatment naïve PWID. The cohort was grouped into anti-HCV seropositive PWID with detectable HCV-RNA, anti-HCV seropositive PWID without detectable HCV-RNA and anti-HCV seronegative PWID. Although longitudinal data are not available, the anti-HCV seropositive group without detectable HCV-RNA putatively consists predominantly of PWID who spontaneously resolved HCV infection whereas the HCV-RNA positive group predominantly represents PWID with chronic HCV infection. Importantly, based on the experience from previous studies, exposure to HCV may also be common in the group of anti-HCV seronegative PWID with high-risk behavior(6, 7). Although exposure to HCV is difficult to prove in the absence of serological markers for infection, there is growing evidence that the anti-HCV seronegative group is immunologically distinct and may therefore be able to achieve control of viral replication prior to detectable humoral immune responses(8-10).

Here, the genetic association study suggested two distinct genetic predispositions resulting in either preferential spontaneous resolution of hepatitis C or in a protective state against HCV infection prior to seroconversion. KIR3DL1 combined with a high copy number of HLA-Bw4 alleles - defined here as two alleles or more - was associated with an anti-HCV seronegative state. This is consistent with a functional advantage and a protective effect of this genetic constellation upon exposure to HCV at a time prior to seroconversion. Notably, in a group of 179 healthy donors the frequency of individuals with ≥ 2 Bw4 copies (28.5%) was between the frequency of PWID with detectable HCV-RNA (15.2%) and anti-HCV seronegative PWID (39.2%) and was in a similar range compared to anti-HCV seropositive PWID with undetectable HCV-RNA (29.7%). Accordingly, compared to healthy controls individuals with high HLA-Bw4 copy numbers were less frequent in the group of PWIDs with persistent infection and enriched in the anti-HCV seronegative group. In addition, carriage of an HLA class I-allele encoding a Bw4 80(T) motif

combined with KIR3DL1 was significantly enriched among PWID with spontaneous clearance of HCV infection but not in the group of anti-HCV seronegative PWID. The beneficial effect of KIR3DL1 in combination with Bw4 80(T) in clearing HCV after seroconversion was confirmed in an independent cohort of PWID recruited in North America. This suggests a beneficial effect of KIR3DL1 expressing NK cells in the context of its HLA-Bw4 80(T) ligand during acute HCV infection in PWID but no protective effect against HCV at a stage prior to seroconversion.

It has been described that the genetic combination of KIRs and its HLA class I-ligands (homozygous, heterozygous or absent) can influence the functionality of NK cells(27). While for KIR2DL3 and KIR2DL1 expressing NK cells homo- and heterozygosity for the relevant HLA class I-ligand seemed to result in equally potent “arming”(28), KIR3DL1 expressing NK cells from Bw4 homozygous patients are more efficient IFN γ producers compared to NK cells from Bw4 heterozygous or Bw6 homozygous patients(29). In a recent study by Boudreau et al. detailed analysis of the mechanisms underlying the licensing of KIR3DL1⁺ NK cells in a cohort of healthy blood donors suggest that both the binding affinity and the cell surface density of receptor-ligand pairs correlate with the magnitude of NK cell functionality (30). Our results in healthy individuals confirm these findings and collectively support a correlation between the HLA-Bw4 copy number and the functionality of KIR3DL1⁺ NK cells. Notably, the same trend with increased functionality of KIR3DL1⁺ NK cells in the presence of multiple HLA-Bw4 copy numbers was also observed in PWID, although here the differences were not statistically significant.

In our study, Bw4 motifs in HLA-B alleles as well as in HLA-A alleles(31) were considered for the analysis. Accordingly, up to four alleles encoding a Bw4 motif were possible in one individual. Notably, all Bw4 motifs encoded at the HLA-A locus and the majority of the HLA-B alleles encoding Bw4 motifs carry isoleucine in position 80. When Bw4 80(I) positive individuals are compared to Bw4 80(T) positive individuals, there is a possible bias towards individuals with multiple Bw4 alleles in the Bw4 80(I) group.

To exclude such bias only individuals with a single HLA-Bw4 allele were included in the analysis of NK cell function. Interestingly, we found an advantage of HLA-Bw4 80(T) alleles over HLA-Bw4 80(I) alleles regarding NK cell functionality in PWID but not in healthy donors. It is generally believed that HLA class I-molecules carrying the HLA-Bw4 80(I) motif have a higher affinity to KIR3DL1 compared to HLA-Bw4 80(T) molecules and thereby mediate stronger inhibitory signals to NK cells(32-34). Notably, it was reported that the functionality of KIR3DL1⁺ NK cells from healthy donors in the context of HLA-Bw4 80(I) was even better compared to HLA-Bw4 80(T)(30). The reasons for the restricted superior functionality of KIR3DL1⁺ NK cells in the context of HLA-Bw4 80(T) to the PWID group are unclear. It seems possible, that the altered functional NK cell phenotype was adopted during HCV infection. For example, one of the most consistent findings is increased frequencies of NKG2A⁺ NK cells in chronic HCV infection (reviewed in (35)). Yet there is no clear longitudinal data showing if those high NKG2A frequencies are induced by HCV infection or if high frequencies of NKG2A⁺ NK cells prior to HCV transmission promoted persistence of infection. A recent study by Krämer et al. reports similar differences between NK cells of HCV-RNA positive individuals and healthy controls. In this study patients were grouped according to their IL28B genotype for NK cell functionality analysis. While NK cells of HCV-RNA positive individuals with an IL28B TT-genotype were functionally impaired when compared to HCV-RNA positive individuals with an IL28B CC- or CT-genotype, no corresponding difference was found in healthy individuals (36). Taken together with our findings, this may indicate that HCV exposure itself perturbs the NK cell compartment in a more complex way than previously appreciated.

Previous studies revealed an association between KIR2DL3 combined with homozygosity for its ligand HLA-C1 and spontaneous clearance of hepatitis C as well as a protective state in anti-HCV seronegative PWID(8, 19-22); however, partially conflicting results were observed in different cohorts and distinct “protective” KIR/KIR-ligand associations have been reported in some studies(23, 24). Of note, while on the genetic level we do not see a beneficial effect for KIR2DL3 and HLA-C1 in our German PWID cohort,

we have previously reported that, on a phenotypic level, higher expression of KIR2DL3 is associated with spontaneous immune control of HCV and protection from infection (10). Collectively, the existing data from genetic association studies support the idea that the genetic correlates for “protection” against HCV infection are not universal and that the pathways to immune control involve multiple discrete factors(37). This also includes the genetic determinants of the CD8⁺ T cell response, that plays an important role in antiviral immunity against hepatitis C. In particular, presence of HLA-B*27 and HLA-B*57 was reproducibly associated with spontaneous immune control of HCV infection(38-41). Notably, in our cohort of PWID HLA-B*27:05 was also associated with a protective effect against HCV (data not shown). As HLA-B*27:05 carries the HLA-Bw4 80(T) motif, we excluded, the possibility that the protective effect of the KIR3DL1/HLA-Bw4 80(T) genotype was driven solely by HLA-B*27 as, after removal of all HLA-B*27 positive patients the KIR3DL1/HLA-Bw4 80(T) genotype remained more frequent in the group of HCV-RNA negative PWID (37.5%) compared to HCV-RNA positive PWID (22.9%; p=0.05). While we have demonstrated a protective influence of KIR3DL1/HLA-Bw4 80(T) on spontaneous resolution of hepatitis C in two large cohorts, this was not observed in our UK population. This is likely to be multifactorial. However, we have performed a further analysis on our cohorts. This shows that the UK cohort has a different frequency of HLA-B allotypes compared to the German cohort. Specifically, there was an over-representation of HLA-B*44 (19.2% vs 9.4%) and HLA-B*57 (4.9% vs 1.7%), with lower frequencies of HLA-B*13 (1.8% vs 4.3%) and HLA-B*38 (0.7% vs 3.2%). The relative balance between resolution by cytotoxic lymphocytes and resolution through NK cells may thus be different between the two populations. Moreover, additional factors that influence the outcome of HCV-infection such as sex (male/female), viral genotype, transmission route and infection dose at exposure(19, 42) are typically not well controlled in cohorts for genetic association studies and may partially explain discrepant results between cohorts.

Although KIR3DL1 has not been previously described to influence the natural course of HCV infection, the combination of KIR3DL1 and HLA-Bw4 alleles has recently been described to influence treatment outcome. In a Japanese cohort of patients infected with genotype 1 the IL28B genotype and the KIR3DL1/HLA-Bw4 genotype were independent predictors for treatment success upon combination therapy with pegIFN α and ribavirin(43). The combination of KIR3DL1 and HLA-Bw4 has been associated with beneficial effects in other diseases. For example, protection from diffuse large B-cell lymphoma was described in a cohort of Thai patients(44) and better outcome of patients with metastatic colorectal cancer treated with chemotherapy was associated with the KIR3DL1 genotype in combination with its ligand Bw4(45). Moreover, the risk of relapse of acute myeloid leukemia is significantly reduced in patients with the KIR3DL1/HLA-Bw4 80(T) genotype compared to patients with the KIR3DL1/HLA-Bw4 80(I) genotype, consistent with superior functionality of KIR3DL1⁺ NK cells in the presence of HLA-Bw4 80(T) allotypes against leukemic cells(46). In turn, although in HIV-infection the KIR3DL1/HLA-Bw4 genotype has also been associated with low viral load and slower disease progression to AIDS, this protective effect was predominant for the combination of KIR3DL1 with HLA-Bw4 80(I) allotypes and was not detected or substantially weaker for the combination with HLA-Bw4 80(T) allotypes (reviewed in(47)). Interestingly, in the presence of HLA-Bw4 the KIR3DL1*004 allotype, which lacks KIR3DL1 expression at the cell surface, was also associated with delayed disease progression in the context of HIV infection(48, 49). This strongly suggests that the impact of different combinations of KIR3DL1 allotypes and their ligand HLA-Bw4 allotypes differ between HIV and HCV.

Taken together our data indicate that multiple Bw4 copies are associated with a protective state against HCV in a high-risk group and promote HCV clearance prior to seroconversion, probably mediated by better NK cell licensing. In addition, our genetic data indicate a second protective mechanism of KIR3DL1⁺ NK cells in the presence of HLA-Bw4 80(T) allotypes that acts during acute HCV infection and promotes spontaneous resolution; but does not prevent anti-HCV seroconversion. Although future

longitudinal studies are required to specifically address the impact of these genetic constellations on the outcome of hepatitis C, we suggest that superior functionality of NK cells against HCV plays a mechanistic role for the advantageous effect of high HLA-Bw4 copy numbers and the KIR3DL1/HLA-Bw4 80(T) genotype on the natural outcome of HCV infection in a high-risk group.

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Figure legends

Figure 1: Frequency of the KIR3DL1/Bw4 80(T) genotype in Germany and North America according to the infection status. The frequency of individuals with the KIR3DL1/Bw4 80(T) genotype in HCV-RNA positive PWID (light gray) and HCV-RNA negative PWID (dark gray) are shown in percent. P-values were calculated by Fisher's exact test.

Figure 2: 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 individuals. (B) IFN γ producing KIR3DL1⁺ NK cells upon stimulation with K562 target cells 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(26) (Kruskal-Wallis test , * $p \leq 0.05$ and *** $p < 0.001$).

Figure 3: 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 IFN γ producing (A+D), TNF α -producing (B+E) and CD107a (C+F) expressing KIR3DL1⁺ NK cells according to the number of Bw4 motifs are shown (Kruskal-Wallis test or One-way ANOVA , * $p \leq 0.05$ and *** $p < 0.001$).

Figure 4: 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. Horizontal lines indicate the median. The frequency of IFN γ producing (A+D), TNF α -producing (B+E) and CD107a (C+F) expressing KIR3DL1⁺ NK cells according to the KIR3DL1-ligand status are shown (Kruskal-Wallis test or One-way ANOVA, * $p \leq 0.05$ and ** $p < 0.01$).

Graphical abstract: After exposure to HCV the majority of PWID develop chronic HCV infection that can lead to liver cirrhosis and hepatocellular carcinoma. A small subgroup of PWID is able to clear HCV infection before and after seroconversion. PWID with multiple Bw4 alleles are more likely to clear HCV prior to seroconversion than PWID with one or no Bw4 allele. In addition PWID that encode both KIR3DL1 and a Bw4 80(T) motif have higher chances of spontaneously clearing HCV after seroconversion.

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Table 1: Patient characteristics PWID

	Germany			North America	
	HCV-RNA positive	HCV-RNA negative	anti-HCV negative	HCV-RNA positive	HCV-RNA negative
complete cohort					
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 GT1 (%)	84 (55.6%)	n.d.	n.d.	158 (59.2%)	n.d.
HCV GT3 (%)	64 (42.4%)	n.d.	n.d.	38 (14.2%)	n.d.
other HCV GT 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%)

Table 2: Results of the genetic association between KIR/KIR-ligands and HCV infection status in the German PWID cohort

gene	HCV-RNA pos	HCV-RNA neg	anti-HCV neg	univariate analysis		multivariate analysis		
				p-value ^a	p-value ^b	p-value ^a	OR	95% CI
rs12979860 CC	38.1%	63.6%	23.3%	0.005	0.061	0.012	2.63	1.241-5.582
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

Table 3: Association between HLA-Bw4 copy number and HCV infection status in PWID

	HCV-RNA pos	HCV-RNA neg	anti-HCV neg	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
(Fisher's exact test)

Fig.1

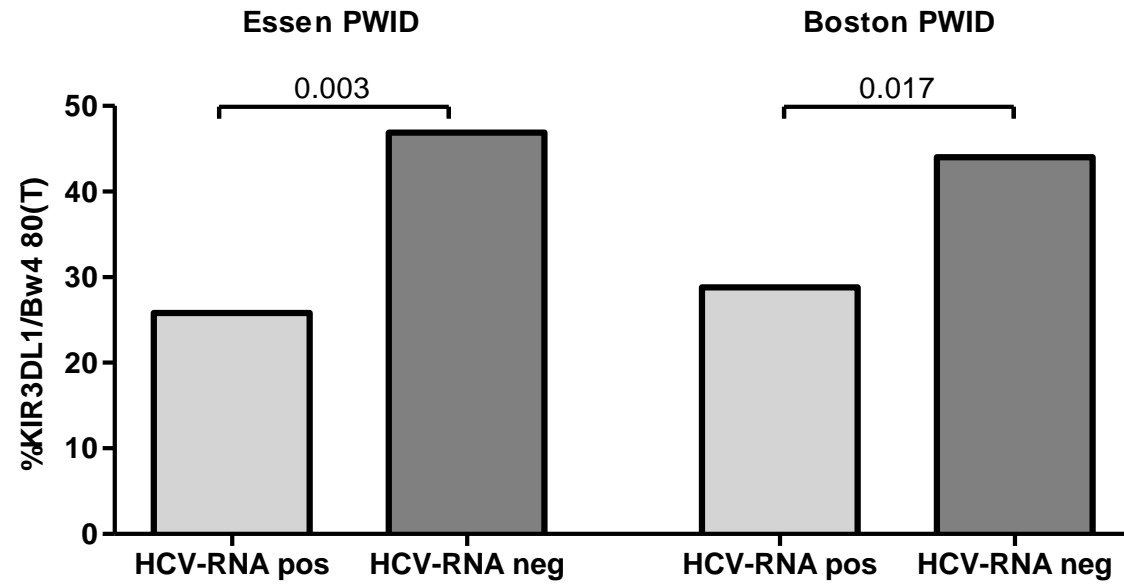
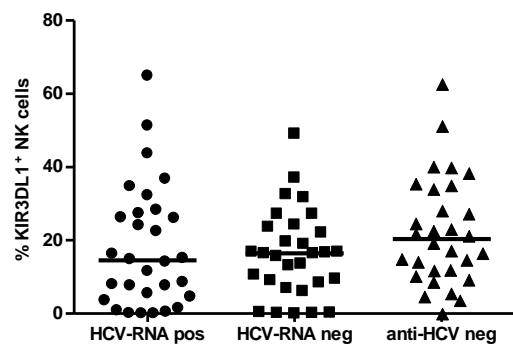
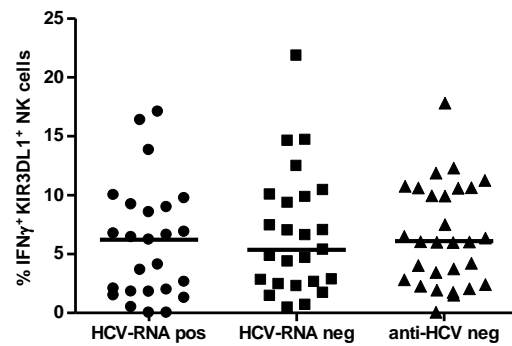


Fig.2

A



B



C

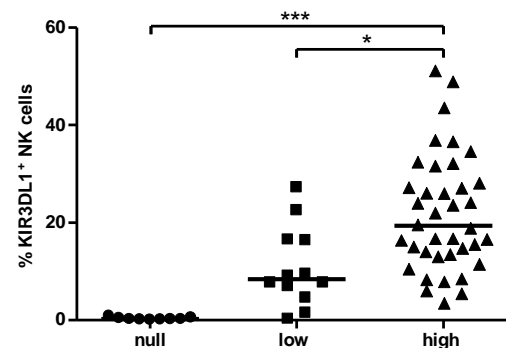


Fig.3

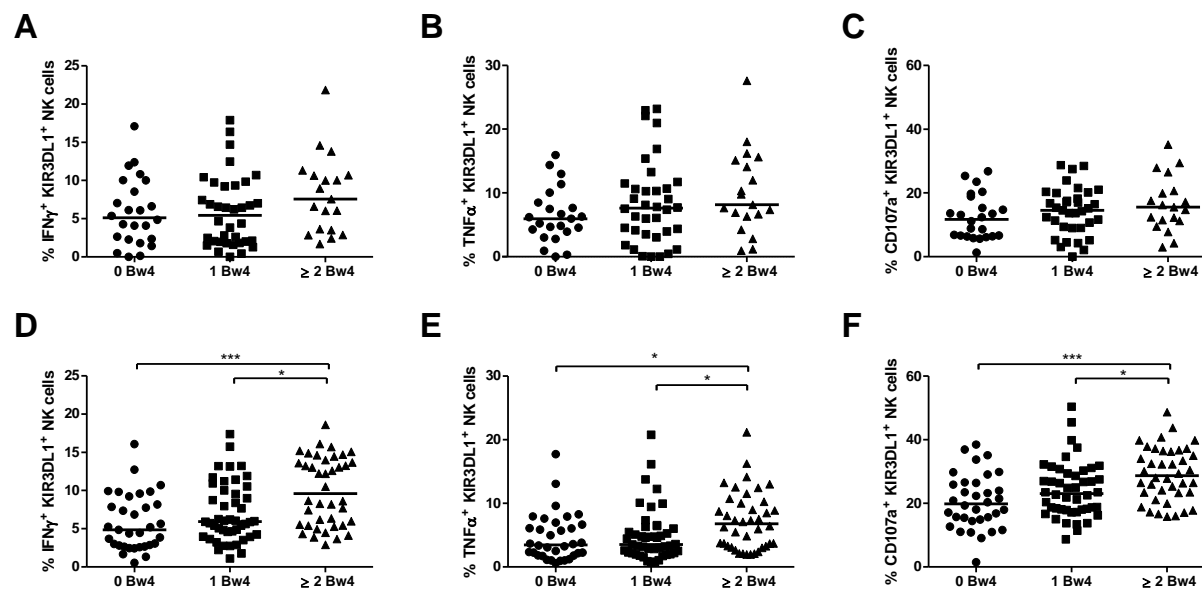
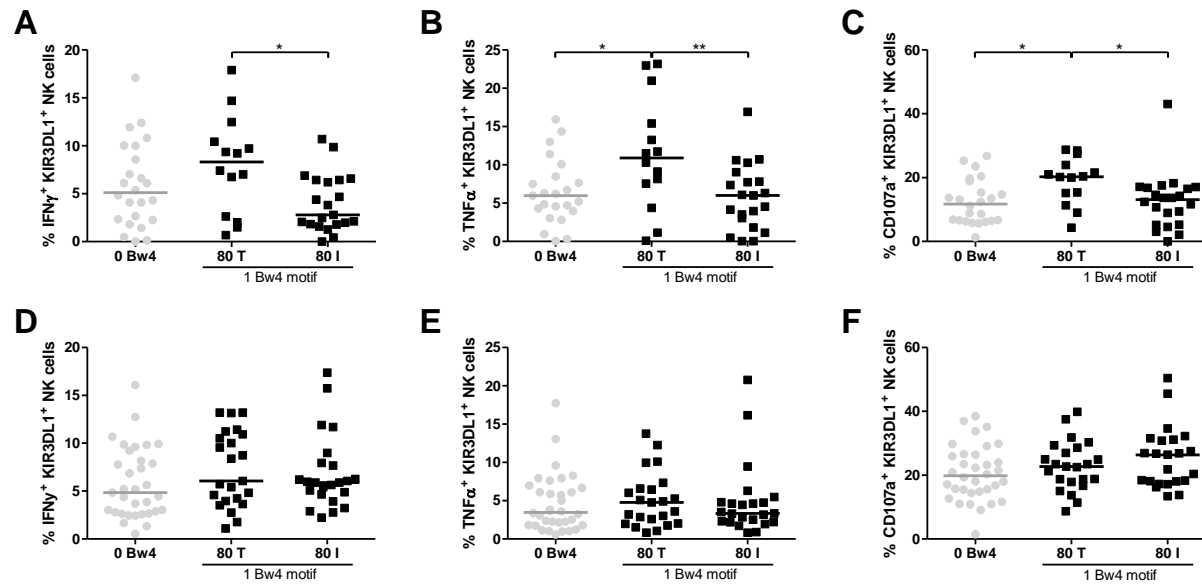
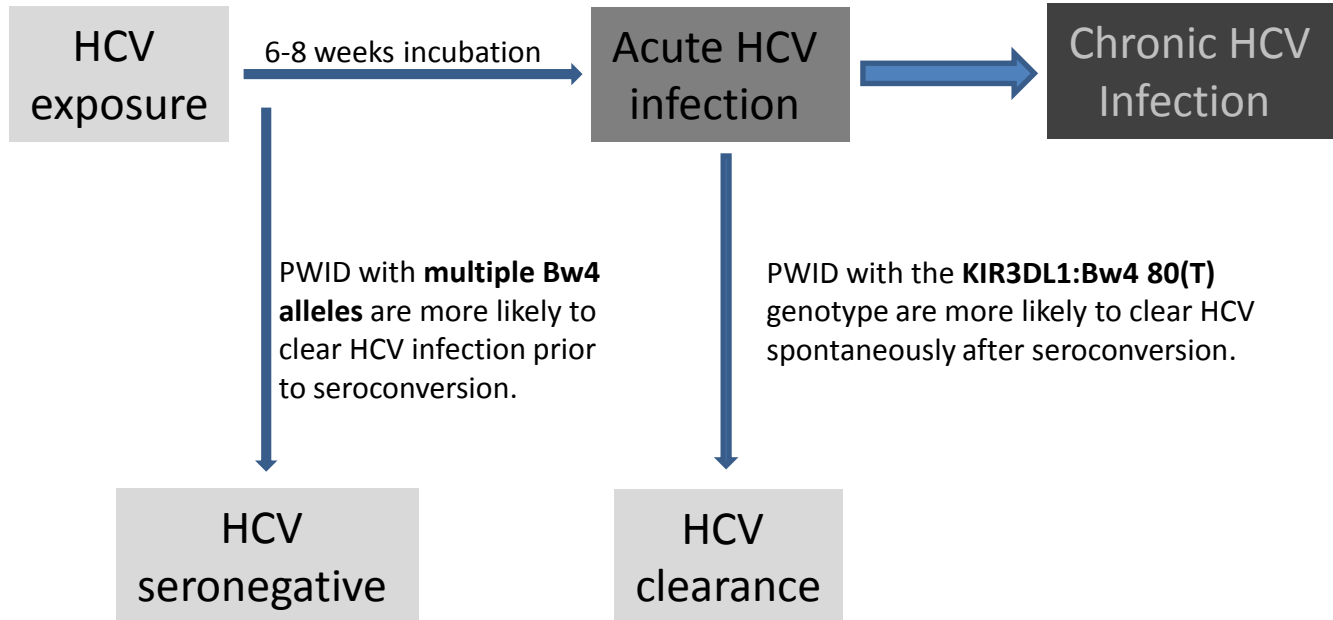


Fig.4





HIGHLIGHTS

- In a high risk cohort of people who inject drugs (PWID) KIR3DL1/HLA-Bw4 80(T) was associated with spontaneous clearance of HCV infection
- Moreover, the frequency of individuals with multiple HLA-Bw4 alleles was significantly higher in anti-HCV seronegative PWID compared to PWID with detectable HCV-RNA consistent with a protective state mediated by HLA-Bw4 alleles upon HCV exposure at a stage prior to seroconversion
- KIR3DL1⁺ NK cells from HLA-Bw4 80(T)-positive PWID showed superior functionality compared to HLA-Bw4 80(I)-positive PWID
- In turn, in healthy individuals the HLA-Bw4 copy number strongly correlated with the functionality of KIR3DL1⁺ NK cells
- HLA-Bw4-80(T) and multiple HLA-Bw4 copies in combination with KIR3DL1 are associated with protection against chronic hepatitis C in PWID by two distinct mechanisms