PHENOTYPE AND FUNCTION OF NATURAL KILLER- AND NATURAL KILLER-LIKE T CELLS IN THE ACUTE PHASE OF HEPATITIS C IN HIV+ PATIENTS

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Pavlos Kokordelis

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1. Gutachter: Prof. Dr. Ulrich Spengler
2. Gutachter: Prof. Dr. Irmgard Förster

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Table of Contents

Summary

Among HIV+ patients an increasing number of people acquire a co-infection with the hepatitits C virus (HCV). Compared to HCV mono-infected patients, the HCV co-infection in HIV+ patients leads to accelerated rates of fibrosis and liver associated morbidity and mortality. Regardless of the pre-existing HIV infection a small proportion of the co-infected patients clear their acute HCV infection naturally in the early stage before establishing a chronic infection. In our studies, we investigated the role of natural killer cells (NK cells) as well as natural killer-like T cells (NK-like T cells) in modulating outcomes of acute hepatitis C infection in HIV+ patients. Patients with chronic HCV/HIV co-infection, HIV mono infection, as well as healthy individuals were studied as controls. NK- and NK-like T cells were studied phenotypically and functionally by flowcytometric analysis. Interferon-gamma (IFN-γ) secretion, degranulation (CD107a) and anti-HCV activity were studied using the Huh7-HCVreplicon cells system. NK- and NK-like T cell frequency did not vary significantly with respect to outcome of acute HCV infection. Nevertheless, *in vitro* NK cells from patients with a self-limited course of HCV infection were able to inhibit HCV replication to a greater extent than NK cells from patients who developed chronic course of infection. Functional analysis showed a positive correlation between NK cell anti-HCV activity and IFN-γ secretion whereas no such observations were made with respect to NK cell degranulation. Blocking of IFN-γ suggested a key role for NK cell-mediated inhibition of HCV replication. In line with this, NK cells from patients with a self-limited course produced significantly more IFN-γ than NK cells from patients with a chronic course. Further we found that high expression of NKp46 and NKG2D defines NK cell subpopulations which display a robust anti-HCV activity and are able to modulate outcomes of acute HCV infection favoring spontaneous clearance via IFN-γ secretion. Blocking of these receptors significantly reduced antiviral NK cell activity. Regarding CD3(+)CD56(+) NK-like T cells from healthy individuals, we found that stimulation with IL-12/IL-15 significantly increased efficacy to inhibit HCV replication *in vitro* via IFN-γ secretion. In line with our previous observations from the NK cell analysis, blocking of IFN-γ decreased anti-HCV activity of NK-like T cells. However, no correlation was found between degranulation and inhibition of HCV replication. Instead, we found NK-like T cells from HIV+ mono infected patients

to display a significantly impaired IFN-γ production and consequently to show significantly worse capacity to inhibit HCV replication than healthy controls.

In conclusion, our results demonstrate a critical role of NK cell subpopulations and their capacity to modulate IFN-γ production as a key factor for a robust anti-HCV response. We could show that NK cell-mediated IFN-γ production was associated with spontaneous clearance of acute hepatitis C in HIV+ patients. Despite the fact that NK-like T cells also possess the ability to inhibit HCV replication in an IFN-γ-dependent manner, their capacity to produce IFN-γ seems to be negatively affected by HIV infection, so that they can contribute only insignificantly to the clearance of hepatitis C virus in HIV infected patients.

1 Introduction

1.1 Hepatitis C Virus (HCV)

Hepatitis C represents an inflammation of the liver caused by the hepatitis C virus (HCV). This parenterally acquired disease, especially through blood transfusion, was initially described as post-transfusion hepatitis, or non-A-non-B hepatitis [1]. In 1989 the group of Choo et al. succeeded in isolating viral RNA [2] and the subsequent characterization of HCV as single-stranded RNA virus with positive polarity (5' -> 3') (ss(+)RNA). Next HCV was assigned to the family of *Flaviviridae.* Until now, seven different genotypes have been determined [3]. Worldwide around 71 Mio people (1%) of the global population, are chronically infected with HCV. WHO estimates that there were approximately 1.75 million new infections in 2015 and around 400000 infected individuals die from HCV every year [4]. Until now, there is no HCV vaccination available.

1.1.1 Transmission

The transmission of the HCV occurs via blood-blood contact. Thus, patients who frequently received blood transfusions or blood products (hemophilia patients) had the biggest risk of infection until the identification of the hepatitis C virus as pathogen causing non-A-non-B hepatitis [5]. Today, people with drug addiction are the most vulnerable risk group. The infection occurs from contaminated drug paraphernalia [6]. The risk of a sexual transmission is generally perceived as low. Nevertheless, over the last ten years a significant increase of new infections can be observed among homosexual men. Besides other causes, this is attributed to an increased risk of infection due to traumatic sexual practices, which can lead to mucous membrane injuries [7].

1.1.2 Clinical course

Since the infection is asymptomatic in most cases, it often remains undiscovered at first. After an incubation period of approx. 8 (2-26) weeks a temporary increase of transaminases occurs. Only a small proportion of patients (10-20%) shows a strong increase of liver enzymes or icterus. During this time, around one third of patients suffers from flu-like symptoms. The chronic infection, on the other hand, is asymptomatic, except for a general fatigue. Only in 20-30% of patients the virus can be eliminated by the endogenous immune response during the acute phase, which usually describes

the first six months of the infection. However, in most cases the acute hepatitis develops into a chronic hepatitis C (CHC). Over the clinical course, the CHC can develop from a simple infection of the liver (mild hepatitis) to a cirrhosis and even to a hepatocellular carcinoma. Around 20% of chronically infected HCV patients develop a liver cirrhosis within 20 years. When cirrhosis is established, the development rate of the hepatocellular carcinoma is around 1-5 % per year [3].

1.2 Human Immunodeficiency-Virus (HIV)

HIV is a RNA virus with positive polarity (5' -> 3') (ss(+)RNA) and belongs to the Family of *Retroviridae.* Until today, two different HIV types are known. HIV-Type1 was discovered in 1983 [8] and HIV-Type 2 in 1986. HIV-Type 1 is distinguished in four subgroups (M, N, O and P), which are again classified in several subtypes. To date, around 60 million of HIV-positive people are infected with HIV-Type1 of the subgroup M, which is also responsible for almost all AIDS cases [9]. In 2016, around 36.7 million people around the world lived with an HIV infection and 1.8 million people became newly infected [10].

1.2.1 Transmission

The transmission via sexual contact is the dominant path of infection, followed by use of contaminated drug paraphernalia. The perinatal transmission from a mother to a child is also possible. On the other hand, infections from blood transfusions or organ transplantations are very rare since the introduction of HIV tests. The risk of infection correlates with the present amount of virus in the blood or sperm and in the vaginal secretion, since the highest virus concentrations are measurable here [11]. The HIV enters the target cell via the CD4 receptor, which is expressed mostly by T cells, but also by monocytes, macrophages and dendritic cells (DC).

1.2.2 Clinical course

After successful entry into the cell, the virus begins to replicate itself, which leads to the reproduction and release of new virus particles, which infect the CD4(+) cells. The CD4(+) T (T helper) cells are the most affected celltype and their number decreases with increasing viral load. In most cases the infection is asymptomatic at first, although in some patients an acute, retroviral syndrome with flu-like symptoms and swelling of lymph nodes can occur. This phase usually lasts for up to four weeks. After this phase,

the number of CD4(+)T cells increases again and the viral load decreases. The immune system stabilizes temporarily through the initiation of a longtime immune response. Without a highly active antiretroviral therapy (HAART), over the course of ten years the clinical course is usually a constant decrease of CD4(+)T cells, an increase of the viral load and the potential occurrence of opportunistic diseases until finally the stage acquired immune deficiency syndrome (AIDS) is reached [11].

1.3 HIV/HCV co-infection

Due to similar transmission pathways, a relevant proportion of HIV patients show a co-infection with the hepatitis C virus. In Germany, around 15%, in other European countries and the USA around 33% of all HIV(+) patients are HCV co-infected [11]. In several studies it could be verified that co-infection with HIV has a negative impact on the course of the hepatitis C infection due to the concomitant compromise of the immune system. Therefore, on average co-infected patients have higher HCV viral loads than people with HCV mono-infection. Furthermore, HIV/HCV co-infection is associated with a faster progression towards liver fibrosis and liver cirrhosis which leads to liver failure and increased mortality [12], [13]. To date, liver failure is the most frequent cause of death in HIV/HCV patients [14]. The treatment is also more complex in co-infected patients, compared to people with a mono-infection. For example, an increase of hepatotoxicity of antiretroviral substances has been observed for HCV infections, where up to 10% of treated patients did not tolerate the therapy and had to change the medication [9].

1.4 Natural Killer cells (NK cells)

NK cells, a sub-population of lymphocytes, are an integral element of the innate immune system. They are characterized by the presence of CD56 (N-CAM) and the absence of the CD3 receptor. Around 90% of circulating NK cells (CD3-CD56dimCD16+) express low amounts of CD56 on their surface. These cells express CD16 (FcγRIII), the receptor of the IgG Fc fragment and exercise strong cytotoxic effects. A second sub-population expresses higher amounts of CD56 but no CD16 (CD3-CD56brightCD16-). Immune regulatory properties such as a distinct IFN-γ production, are associated with the CD56bright subtype [15]. In healthy individuals, they represent 5-15% of the total peripheral mononuclear blood cells (PBMC) and up to 45% of the intrahepatic lymphocytes [16]–[18]. A relevant function of NK cells is the destruction of

transformed or (intracellularly) infected cells, infected from either viruses or bacteria. In contrast to T cells, NK cells are able to trigger an immune response without prior 'priming'. The activity of NK cells is regulated by NK cell receptors (NKR), which can be distinguished in activating or inhibiting NKR. The natural cytotoxicity receptors (NCR), among others, as well as some members of the NKG2 family belong to the activating receptors [19].

1.4.1 Natural cytotoxicity receptors

The activation of natural killer cells is primarily mediated via the natural cytotoxicity receptors NKp30, NKp44 and NKp46 [20], which belong to the Ig superfamily. NKp30 and NKp46 are expressed on both, resting as well as activated NK cells. NKp44 on the other hand, can be found only on cytokine-activated NK cells. NKp46 binds N-acetylneuramine –binding glycoproteins e.g. hemagglutinin, as well as hemagglutinin neuraminidase from influenza and parainfluenza viruses. The natural ligands for NKp30 and NKp44 are not yet known [21], [22].

1.4.2 CD94/NKG2-Receptors

Type II lectin-like, integral membrane glycoproteins give rise to the so-called CD94/NKG2 receptors. On the cell surface NKG2 proteins form heterodimers with CD94, which is the invariant Type II, lectin-like polypeptide. CD94 itself cannot transmit any signals, since it does not have a cytoplasmic tail, which is essential for the surface expression of NKG2 receptors. While some of the NKG2 receptors have an activating function (NKG2C, H and E), others have inhibiting properties (NKG2A and B). The ligand for CD94/NKG2A and CD94/NKG2C is the non-classic MHC I-molecule HLA-E [23]–[25].

1.4.3 NKG2D

NKG2D is the only known member of the NKG2 family that is not expressed via covalent binding to CD94. Its cognate ligands are MICA, MICB and ULBPs, among others, which in terms of structure resemble MHC-I-like proteins. After binding to its ligand, the NKG2D receptor transmits activating signals, which increase the cytotoxicity of NK cells and also trigger the production of IFN-γ [26]–[28].

1.5 NK cells in HCV mono-infection

As part of the innate immune system NK cells play an important role in the defense against pathogens, since they are able to inhibit the viral replication in the early phase of the virus infection as well as to induce a strong adaptive immune response. Regarding hepatitis C, epidemiologic data as well as *in vitro* experiments verify, that NK cells are activated and potentially transmit protection against a HCV infection and can positively affect the natural course of the infection [29]–[32]. It could be demonstrated *in vitro*, that HCV replication can be inhibited via an IFN-γ dependent mechanism by cytokine-activated NK cells [33], [34]. Recently published studies in HCV(+)/ HIV(-) patients show, that an activation of the NK cell population occurs during the acute phase of the HCV infection, independent of the further course of the infection (self-limiting vs chronic). The questions to which degree and via which mechanisms NK cells regulate the immune elimination of the virus could not be finally resolved during the research. However, Alter et al. found that the expression of NKp46 and NKG2D significantly differed between NK cells of patients with self-limiting hepatitis C and patients with a chronic course of infection. This indicates a great importance of NKp46(+) and NKG2D(+) NK cells within the context of the acute hepatitis C [29], [35], [36].

1.6 NK-like T cells and HCV

CD3(+)CD56(+) NK-like T cells are another subpopulation of innate lymphocytes which share several characteristics with NK cells. This includes their large granular lymphocyte morphology, their potency to lyse NK-sensitive target cells as well as their functional regulation, which is mediated by classic NK cell receptors [37]. CD3(+)CD56(+) NK-like T cells represent approximately 5%–15% of the peripheral T cell pool and up to 50% of T cells in the liver, which must be considered as the main site of HCV replication [38]. CD3(+)CD56(+) NK-like T cells are co-expressing NK cell markers, capable of leading to either suppression or stimulation of immune responses [37]. Because of the fact that CD3(+)CD56(+) NK-like T cells can mediate both MHC-restricted and MHC-unrestricted cytotoxicity as well as cytokine production, they show properties of both T and NK cells [39], [40]. Phenotypic and functional alterations of CD3(+)CD56(+) NK-like T cells have been revealed to be associated with the outcome of acute hepatitis C in HIV-positive patients [41], indicating that this lymphocyte subset might influence the immunopathogenesis of HCV infection. Furthermore the role of

CD3(+)CD56(+) NK-like T cells in hepatitis C manifests itself by the detection that in the livers of patients with chronic HCV infection the numbers of CD3(+)CD56(+) NK-like T cells are notably decreased. This condition may be related to an increased susceptibility to develop hepatocellular carcinoma [42]. Anyway, it has been shown that exposure to HCV is associated with both increased activation and degranulation of NKT cells [43], [44]. However, it is still unclear whether CD3(+)CD56(+) NK-like T cells exert direct activity against HCV. Additionally, it has not been investigated yet if HIV coinfection affects anti-HCV activity of CD3(+)CD56(+) NK-like T cells. Concerning this, it is necessary to consider that HIV infection has been revealed to be associated with a decrease in CD3(+)CD56(+) NK-like T cells number and morphological and functional changes of CD3(+)CD56(+) NK-like T cells [45], [46].

1.7 Aim of the thesis

This present work focuses on the exploration and research of the role of natural killer cells (NK cells), as well as natural killer-like T cells (NK-like T cells) during the acute Hepatitis C virus infection in HIV(+) patients. The experiments emphasize both, the intracellular, as well as the extracellular characterization of NK and NK-like T cells, and their functions. Here, the analysis addressed the expression of the activating and inhibiting receptors as well as the maturation markers on NK and NK-like T cells. Further, the cytotoxicity (CD107a), antiviral activity (Luciferase) as well as cytokine profile (IFN-γ) of NK and NK-like T cells, were investigated. These investigations help to understand which mechanisms are involved in the spontaneous clearance of hepatitis C in its early stage in HIV+ patients.

2 Materials

2.1 Devices

Laminar flow cabinet	Microflow GmbH, Germany
Flow cytometer FACSCanto II	BD, Germany
CO_2 incubator	Forma Scientific, Germany
Inverted microscope (DM/LS)	Leica Microsystems, Germany
Cooling centrifuge	Beckman Coulter, Germany
Benchtop centrifuge	MIKRO 22R Hettich, Germany
Luminescence reader	Wallac, USA
OctoMACS separator	Miltenyi, Germany
Elisa reader	Tecan, Switzerland
Shaker	VWR, Germany
Vortex	Scientific Idustries, USA
Neubauer chamber	Brand, Germany

2.2 Disposable materials

Polypropylene reaction tube (1.5µl)	Eppendorf, Germany
Polypropylene tube (14, 50 µl)	Greiner Bio-One, Germany
Pipettes (5, 10, 25 ml)	Greiner Bio-One, Germany
Pasteur pipettes	Brand, Germany
Pipettes (10, 100, 200, 1000ml)	Eppendorf, Germany
Disposable gloves	Ansell, Belgium
Microscope slides	Menzel, Germany
Coverslips	Menzel, Germany
S-Monovette for heparin whole blood	Sarstedt, Germany
Cell culture plates (96-, 48-, 24- well)	Greiner Bio-One, Germany
Cell culture flasks (25, 75, 175 cm^2)	Greiner Bio-One, Germany
Polystyrene FACS tubes	Sarstedt, Germany
MACS columns	Miltenyi, Germany
96-well white solid plate	Corning Incorporated, USA

2.3 Media, Buffers and Kits

RPMI 1640 Medium with L-Glutamin	PAA Laboratories, Austria
DMEM Medium with L-Glutamin	PAA Laboratories, Austria
Fetal calf serum (FCS)	Biochrom, Germany
Penicillin/Strepromycin	PAA Laboratories, Austria
Phosphate-buffered saline (PBS)	Pharmacy Univ. Hospital Bonn, Germany
Ampuwa (sterile Aqua dest.)	Fresenius, Germany
Ethanol absolute	Merck, Germany
Accutase	PAA Laboratories, Austria
Dimethyl sulfoxide (DMSO)	Merck, Germany
Ficoll (Lymphocyte separation Medium)	PAA Laboratories, Austria
Cytofix/Cyroperm™	BD, Germany
CellFIX™	BD, Germany
Trypan blue	Merck, Germany
Brefeldin-A (BFA)	Biolegend, USA

Golgi-Stopp	BD, Heidelberg
Steady Glo	Promega, USA
Recombinant human IL-2	R&D Systems
Recombinant human IL-12	R&D Systems
Recombinant human IL-15	R&D Systems
96-well human IFN-γ ELISA kit	Diaclone, USA
NK Cell Isolation Kit, human	Miltenyi, Germany
$CD3^+CD56^+$ NKT Cell Isolation Kit, human	Miltenyi, Germany

2.4 Antibodies

Antibody	Clone	Isotype	Manufacturer
Anti-CD3-APC-Cy7	UCHT1	Mouse IgG1 κ	Biolegend
Anti-CD3-PerCP	SK7	Mouse IgG1 κ	Biolegend
Anti-CD27-PE	O323	Mouse IgG1 κ	Biolegend
Anti-CD56-APC	HCD56	Mouse IgG1 κ	Biolegend
Anti-CD57-APC	HCD57	Mouse IgM κ	Biolegend
Anti-CD62L-FITC	DREG-56	Mouse IgG1 κ	Biolegend
Anti-CD69-PE	298614	Mouse IgG_{2A}	R&D Systems
Anti-CD107a-PE	H4A3	Mouse IgG1 κ	BD Pharmingen
Anti-CD127-FITC	A019D5	Mouse IgG1 κ	Biolegend
Anti-CD161-FITC	HP-3G10	Mouse IgG1 κ	Biolegend
Anti-CD158e-FITC	DX9	Mouse IgG1 κ	Biolegend
Anti-IgG1-PE	MOPC-21	Mouse IgG1 κ	BDPharmingen
Anti-IgG1-PE	RMG1-1	Rat IgG1	Biolegend
Anti-IgG1-FITC	RMG1-1	Rat IgG1	Biolegend
Anti-IgG1-PE	97924	Mouse IgG1	R&D Systems
LEAF Anti-IgG1	MOPC-21	Mouse IgG1	Biolegend
Anti-NKG2A-PE	131411	Mouse IgG_{2A}	R&D Systems
Anti-NKG2C-PE	134591	Mouse IgG1	R&D Systems
Anti-NKG2D-PE	149810	Mouse IgG1	R&D Systems
Anti-NKp46-FITC	9E2	Mouse IgG1	Biolegend
Anti-IFN-γ-PE	25723	Mouse IgG_{2B}	R&D Systems
Anti-NKG2D (block)	149810	Mouse IgG1	R&D Systems
Anti-NKp46 (block)	No indication	Goat IgG	R&D Systems
Anti-IFN-γ (block)	NIB42	Mouse IgG1	Biolegend
LEAF Anti-IFN-γ (ELISA)	NIB42	Mouse IgG1 κ	Biolegend

2.5 Media composition

2.5.1 Lymphocyte Medium

RPMI 1640 Medium with L-Glutamine

10% FCS

1% Penicillin/Streptomycin

2.5.2 Huh7-HCVreplicon cells Medium

DMEM Medium with L-Glutamine

10% FCS

1% Penicillin/Streptomycin

2% G418

1% NEA

0.06% Blasticidin S

2.5.3 Cryoprotectant Medium

RPMI 1640 Medium with L-Glutamine

10% FCS

10% DMSO

2.6 Buffer

2.6.1 MACS-Buffer

PBS

0.5% BSA

2mM EDTA

3 Methods

3.1 Patients

Blood samples were collected from HIV-positive patients with acute hepatitis C and different outcomes of the HCV infection. For the NK cell project, 27 patients with acute hepatitis C and different outcomes of HCV infection were analyzed (Table 1). For the NK-like T cell project, 36 patients with acute hepatitis C and different outcomes of HCV infection were analyzed (Table 2). As controls, 12 patients with HIV/HCV chronic infection, 8 HIV mono-infected patients as well as 12 healthy individuals (HIV-/HCV-) were studied. The samples were collected during the acute hepatitis C diagnosis, from the HIV outpatient clinic from the Bonn University hospital as well as from the Praxis am Ebertplatz, Cologne. For the NK cell study, all HIV-positive patients were HIV RNA-negative under effective HAART. In the NKT cell study, 9 out of 36 aHCV/HIV-positive co-infected patients had a HIV viremia <27.000 copies/ml. Informed consent was obtained from all patients. The study had been approved by the local ethics committee of the University of Bonn (Bonn, Germany).

Table 1. Patient Characteristics

	HIV(+) / aHCV		HIV(+) / cHCV	HIV(+) / HCV(-)	healthy controls
	acute -> self-limited	acute -> chronic			
Number	10	17	12	8	12
Male sex [a]	10 (100%)	17 (100%)	11 (92%)	6 (75%)	7 (58%)
Age (years) [c]	39.1 (31-46)	41.6 (34-49)	47.8 (38-58)	51 (35-66)	30.4 (21-54)
Risk Factors					
MSM [a),b)]	9 (90%)	16 (94%)	6 (50%)	6 (75%)	-
Others/Unknown [a]	1 (10%)	1 (6%)	6 (50%)	2 (25%)	-
Clinical data					
ALT U/L [c]	742 (23 - 3208)	651 (26 - 2262)	70 (4 - 181)	26 (16 - 40)	n.a. [d]
AST U/L [c]	493 (16 - 1638)	348 (24 - 2006)	49 (20 - 102)	20 (16 - 24)	n.a. [d]
HIV-Status					
HIV RNA negative (under HAART) [a]	10 (100%)	17 (100%)	12 (100%)	8 (100%)	-
CD4 cells /µl [c]	647 (358 - 873)	698 (300 - 1377)	620 (182 - 1151)	737 (421 - 1199)	n.a. [d]
CD8 cells /µl [c]	1205 (407 - 1794)	1118 (220 - 2775)	631 (254 - 2249)	1128 (366 - 1940)	n.a. [d]
HCV-Status					
HCV Load (x 10^6 IU/mL) [c]	15.9 (<0.1 -69)	10.7 (<0.1 - 69)	4.9 (0.8 - 26)	-	-
HCV-Genotypes:					
Genotype 1 [a]	8 (80%)	12 (71%)	10 (83%)	-	-
Genotype 2 [a]	0 (0 %)	0 (0 %)	0 (0 %)	-	-
Genotype 3 [a]	0 (0 %)	0 (0 %)	0 (0 %)	-	-
Genotype 4 [a]	0 (0 %)	5 (29 %)	2 (17 %)	-	-
Undetermined Genotype [a]	2 (20%)	0 (0 %)	0 (0 %)	-	-

[a] number of cases (number/total in %)

[b] MSM; men who have sex with men

[c] mean (range)

[d] na - not analyzed

Table 2. Patient Characteristics

	HIV(+) / aHCV		HIV(+) / cHCV	HIV(+) / HCV(-)	healthy controls
	acute -> self-limited	acute -> chronic			
Number	13	23	12	8	12
Male sex [a]	13 (100%)	23 (100%)	11 (92%)	6 (75%)	7 (58%)
Age (years) [c]	39 (31-50)	41.22 (27-49)	47.8 (38-58)	51 (35-66)	30.4 (21-54)
Risk Factors					
MSM [a),b)]	11 (85%)	21 (91%)	6 (50%)	6 (75%)	-
Others/Unknown [a]	2 (15%)	2 (9%)	6 (50%)	2 (25%)	-
Clinical data					
ALT U/L [c]	721 (23 - 3208)	629 (21 - 3644)	70 (4 - 181)	26 (16 - 40)	n.a. [d]
AST U/L [c]	459 (16 - 1638)	345 (24 - 2006)	49 (20 - 102)	20 (16 - 24)	n.a. [d]
HIV-Status					
HIV RNA negative (under HAART) [a]	11 (85%)	16 (70%)	12 (100%)	8 (100%)	-
CD4 cells /µl [c]	670 (358 - 904)	627 (300 - 1377)	620 (182 - 1151)	737 (421 - 1199)	n.a. [d]
CD8 cells /µl [c]	1221 (407 - 1794)	1105 (220 - 2775)	631 (254 - 2249)	1128 (366 - 1940)	n.a. [d]
HCV-Status					
HCV Load (x 10^6 IU/mL) [c]	14.7 (<0.1 -69)	11.2 (<0.1 - 69)	4.9 (0.8 - 26)	-	-
HCV-Genotypes:					
Genotype 1 [a]	8 (62%)	15 (65%)	10 (83%)	-	-
Genotype 2 [a]	0 (0 %)	0 (0 %)	0 (0 %)	-	-
Genotype 3 [a]	0 (0 %)	0 (0 %)	0 (0 %)	-	-
Genotype 4 [a]	0 (0 %)	7 (31 %)	2 (17 %)	-	-
Undetermined Genotype [a]	5 (38%)	1 (4 %)	0 (0 %)	-	-

[a] number of cases (number/total in %)

[b] MSM; men who have sex with men

[c] mean (range)

[d] na - not analyzed

3.2 Isolation of peripheral blood mononuclear cells (PBMC)

PBMCs were separated from heparinized whole blood by the method developed by Böyum [47]. For this purpose the whole blood is applied to a Ficoll density gradient. Ficoll is a sugar polymer with a density of 1.077 g/cm^3. Due to their specific density, monocytes and lymphocytes remain as a milky cloud above the gradient, while erythrocyte granulocyte agglomerates pass through the polymer and are pelleted in the sediment.

Procedure:
- Place 15 ml of Ficoll (D = 1.077) in a sterile 50 ml Falcon tube
- Carefully overlay the Ficoll with the heparinized blood
- Centrifuge 20 min, 900 * g, RT, without brake
- Remove milky lymphocyte ring, into a new 14 ml Falcon Tube
- Wash with PBS
- Centrifuge 10 min, 450 * g, RT
- Remove supernatant
- Resuspend the pellet in 14 ml of PBS
- Centrifuge 10 min, 300 * g, RT
- Remove supernatant
- Resuspend the pellet in cryoprotectant medium

3.3 Cell count

Cell count is carried out according to the method developed by Neubauer. Ten µl of trypan blue cell suspension (1:1) are added to a counting chamber, which is closed with a cover glass. Trypan blue is a vital dye in order to distinguish dead and alive cells. If the cells are dead and their membrane is no longer intact, the dye penetrates them and stains the cytoplasm of the cell blue. Only living cells are counted during the cell count. The counting area consists of four large squares and each one contains 16 small squares. The number of cells is calculated according to the following formula:

Number of cells in 16 squares x dilution factor 2 x chamber factor 10 x total volume of the cell suspension in µl

3.4 Flow cytometry

Flow cytometry allows for the simultaneous determination of different cell properties, such as size, granularity and the expression of extra- and intracellular molecules. The flow cytometry is based on the following principle: the examined cells are taken up by a carrier liquid and individually pass a laser beam. When light hits the cell, it is scattered and re-emitted. The forward light measured here (Forward Scatter/ FSC) allows for a statement on the size and the sideways light (Sideward Scatter / SSC) gives information on the granularity of the cells.

For fluorescence-activated cell sorting (FACS) the following antibodies were used: anti-CD3-PerCP, anti-CD3-APC-Cy7 anti-CD56-APC, anti-CD27-PE, anti-CD57-FITC, anti-CD62L-FITC, anti-CD127-FITC, anti-CD158e-FITC, anti-CD161-FITC and anti-NKp46-FITC (Biolegend, Fell, Germany); anti-CD69-PE, anti-NKG2A-PE, anti-NKG2C-PE, anti-NKG2D-PE and anti-IFN-γ–PE (R&D Systems, Wiesbaden-Nordenstadt, Germany); anti-CD107a-PE (BD Biosciences, Heidelberg, Germany) and eFluor670 (eBioscience, Frankfurt, Germany). The samples were analyzed on a FACSCanto II flow cytometer using the CellQuest Pro (BD Biosciences) and Flowjo 7.5 software packages (TreeStar Inc., Ashland, OR).

3.5 Phenotyping of NK and NK-like T cells

In order to determine the NK and NK-like T cell phenotype, the cells are stained with several specific antibodies. The dye helps to identify the percentage as well as the relative fluorescence intensity (RFI) of the surface expression of these antigens. NK cells are defined as CD3-/CD56+ cells and the NKT cells as CD3+/CD56+ cells. All cells located above the isotype control are considered to be positive. The RFI is calculated by the following formula:

RFI= (median observation – median isotype control) / median isotype control

Procedure:

- Thaw cells in water bath
- Wash with PBS
- Centrifuge for 10min, 300*g, RT
- Discard supernatant
- Re-suspend in RPMI 10% FCS 1% P/S

- Determine cell number (Neubauer chamber)
- Transfer 200,000 cells each into 3 FACS tubes
- Surface dye with

for all batches 5 µl anti-CD56-APC
1 µl anti-CD16-PerCP
5 µl anti-CD3-APC-Cy7
1 µl DAPI (20 sec. before measurement)

tube allocation:

1: 5 µl anti-CD161-FITC, 5 µl anti-NKG2C-PE
2: 5 µl anti-CD62L-FITC, 5 µl anti-NKG2D-PE
3: 5 µl anti-CD158e-FITC
4: 5 µl anti-NKp46-FITC, 5 µl anti-NKG2A-PE
5: 5 µl anti-CD57-FITC, 5 µl anti-CD69-PE
6: 5 µl anti-CD127-FITC, 5 µl anti-CD27-PE
7: 5 µl anti-IgG1-FITC, 5 µl anti-IgG1-PE

- incubate in the dark for 20 mins
- wash with PBS
- centrifuge for 10 min, 300*g, RT
- take up cells with cellfix
- measure immediately or store in the dark at 4°C for up to 24 h and then measure

3.6 NK cell and NKT cell separation

Natural killer or natural killer-like T cells were immunomagnetically separated from total peripheral blood mononuclear cells (PBMC) using MACS cell separation technology, and applying the manufacturer`s recommendations (Miltenyi Biotec, Bergisch Gladbach, Germany). The NK cells separation was done by a negative selection. This means that all non-NK cells, i.e. B cells, T cells, dendritic cells, granulocytes, monocytes and erythroid cells were labeled with a cocktail of biotin-coupled antibodies against specific surface molecules. Then a secondary antibody which is conjugated on magnetic particles binds the biotin group of the primary antibody (anti-biotin). Following this procedure labeled cells are then transferred to MACS columns which are placed in a magnetic field. All labeled cells with magnetic beads are retained in the column, while the unlabeled cells pass through the magnetic field. The NK-like T cells separation was provided by a positive selection in two steps. First NK cells were labeled with

a cocktail of biotin conjugated primary antibodies against surface molecules and secondary antibodies with magnetic anti-biotin microbeads. All labeled cells were then depleted by separation through a column placed in a magnetic field. In the second step, CD56 microbeads were used to positively isolate CD3+CD56+ NKT cells from the pre-enriched NKT cell population from the first step. The purity of NK and NKT cells was >95%. Purified NK or NK-like T cells were cultivated in RPMI1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C and 5% CO2.

Procedure for NK cell separation:
- Resuspend 10^7 PBMCs in 40 µl MACS buffer
- Add 10 µl of the NK cell biotin antibody cocktail
- Incubate for 10 min at 4 ° C
- Add 30 µl of MACS buffer and resuspend
- Add 20 µl of the NK cell microbead cocktail
- Incubate for 15 min at 4 ° C
- Wash with MACS buffer
- Centrifuge 10 min, 300 * g, 4 ° C
- Resuspend in 500 µl MACS buffer
- Prepare MACS columns by rinsing with 500 µl MACS buffer
- Add the suspension to the columns and let it pass completely
- Wash columns three times with 500 µl MACS buffer each time
- Wash with PBS
- Centrifuge 10 min, 300 * g, 4 ° C
- Resuspend in 1 ml of RPMI 10% FCS, 1% P / S

Procedure for NKT cell separation:
- Resuspend 10^8 PBMCs in 40 µl MACS buffer
- Add 100 µl of the CD3+CD56+ NKT cell biotin antibody cocktail
- Incubate for 10 min at 4 ° C
- Wash with MACS buffer
- Centrifuge 10 min, 300 * g, 4 ° C
- Resuspend in 400 µl MACS buffer
- Add 100 µl of anti-biotin microbeads
- Incubate for 15 min at 4 ° C

- Wash with MACS buffer
- Centrifuge 10 min, 300 * g, 4 ° C
- Resuspend in 500 µl MACS buffer
- Prepare LD MACS columns by rinsing with 2 ml MACS buffer
- Add the suspension to the columns and let it pass completely
- Wash columns two times with 2 ml MACS buffer each time
- Collect total effluent (pre-enriched NKT cell fraction)
- Centrifuge 10 min, 300 * g, 4 ° C
- Resuspend in 400 µl MACS buffer
- Add 100 µl of the NK cell microbead cocktail
- Incubate for 15 min at 4 ° C
- Wash with MACS buffer
- Centrifuge 10 min, 300 * g, 4 ° C
- Resuspend in 500 µl MACS buffer
- Prepare MS MACS columns by rinsing with 500 µl MACS buffer
- Add the suspension to the columns and let it pass completely
- Wash columns three times with 500 µl MACS buffer each time
- Place column on a collection tube
- Add 1 ml of MACS buffer onto the columns and immediately flush out the fraction with magnetically labeled CD3+CD56+ NKT cells
- Wash with PBS and resuspend in 1 ml of RPMI 10% FCS, 1% P / S

3.7 Interferon-gamma (IFN-γ) Assay (Flow cytometry)

A part of the immune response is the production of chemical transmitter substances, the cytokines. An important cytokine, which has both, antiviral as well as immune stimulating properties, is IFN-γ. The number of IFN-γ-positive NK and NK-like T cells is determined by flow cytometry in this work. For this purpose, the cells are treated with Brefeldin A (BFA), a Golgi inhibitor. This results in an accumulation of IFN-γ in the cell. Afterwards, the cells are made permeable and stained with a specific IFN-γ antibody. Cells were cultivated for 18 hours in the presence or absence of recombinant human interleukin (rhIL)-12 (1 ng/mL) and rhIL-15 (10 ng/mL). For some experiments IL-2-prestimulated NK cells were co-cultivated for 5 hours with Huh7-HCVreplicon cells at 1:1 effector:target (E:T) ratio at 37°C. Then, brefeldin A (10 mikrog/mL) was added for

another 4 hours or 1 hour of co-cultivation respectively, followed by intracellular staining with anti-IFN-γ mAb and FACS analyses.

Procedure:

- plate 1 x 10^5 cells in 96-well plates twice
-stimulate cells with 1ng/ml IL-12 & 10ng/ml IL-15 for 18 h
- after 18 h, add Brefeldin A
- 5 h incubation at 37°C, 5% CO_2
- resuspend cells thoroughly and transfer cell suspensions to FACS tubes
- Surface staining in FACS tubes with 5 µl anti-CD56-APC and 1 µl anti-CD3-PerCP
- incubate in the dark for 20 min. at RT
- Bring Facs tubes to a total volume of 3 ml by adding PBS and spin down for 10 min., at 300*g
- discard supernatant and resuspend cells in 250 µl Cytofix/Cytoperm
- incubate in the dark for 20 min. at 4°C
- wash cells with 500 µl Perm/Wash
- Centrifuge for 10 min.,300*g, at 4 °C
- Wash cell suspension with 500 µl Perm/Wash
- Spin down for 10 min., 300*g, at 4 °C
- Discard supernatant and resuspend pellet with Perm/Wash
- Add 5 µl IFN-γ PE Ab.
- Incubate cells in the dark at 4 °C
- Wash with 1 ml Perm/Wash
- Centrifuge for 10 min., 300*g, 4 °C
- Discard supernatant and resuspend cell pellet with 150 µl PBS
- Immediately analyse cells or store cells in the dark, at 4 °C for 24 h max.

3.8 IFN-γ Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is a microtiter plate-based assay involving antibodies in the detection and quantification of proteins. The plate surface is coated with a high specificity anti-IFN-γ capture antibody. Samples including antigen are then applied to the wells and the antigen is binding to the capture antibody. Biotinylated anti-IFN-γ antibody is given to the wells, which after addition of its substrate (enzyme- conjugated streptavidin) produces a detectable signal.
After co-incubation of Huh7-HCVreplicon cells with sorted NK or NK-like T cells, overnight supernatants were collected and IFN-γ concentrations were analyzed by enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions.

Procedure:
- Prepare standard curve
- Add 100µl of each sample, control and zero control (Standard diluent)
- Add 50µl of diluted biotinylated anti-IFN-γ
- cover and incubate at room temperature (18 to 25°C) for 2 hours
- Washing steps:
    a) Aspirate the liquid from each well
    b) Dispense 0.3 ml of 1x washing solution into each well
    c) Aspirate the contents of each well
    d) Repeat step b and c two more times
- Add 100µl of streptavidin-HRP solution
- cover and incubate at room temperature (18 to 25°C) for 30 min
    a) Aspirate the liquid from each well
    b) Dispense 0.3 ml of 1x washing solution into each well
    c) Aspirate the contents of each well
    d) Repeat step b and c two more times
- Add 100µl of ready-to-use TMB substrate solution
- Incubate in the dark for 12-15 minutes at room temperature
- Add 100µl of H2SO4: "Stop Reagent"
- Read the absorbance value immediately on a spectrophotometer

3.9 CD107a Degranulation and Killing Assay

CD107a or LAMP-1 (lysosome-associated membrane protein-1) is a functional marker for NK cell activity [48]. CD107a can be found on the inner membrane side of granules in resting NK cells. After activation of the NK cells, the granules fuse with the plasma membrane (exocytosis), and their contents are released into the extracellular space. The CD107a protein is now on the NK cell surface and can be detected with a specific antibody. To prevent the degradation of CD107a by internalization through endocytosis, GolgiStop is added during the co-incubation of NK cells and target cells. GolgiStop is a protein transport inhibitor containing monensin, which prevents acidification of the endosomes and thus a degradation of the CD107a molecule.
Interleukin (IL)-2 (25U/mL)-stimulated PBMCs were coincubated with Huh7-HCVreplicon cells at 1:1 effector:target (E:T) ratio in the presence of anti-CD107a in order to assess degranulation. For the killing assay, isolated NK cells were cultivated with IL-2 (25 U/mL) for 18 hours. Furthermore, NK cells were coincubated with eFluor670-labeled Huh7-HCVreplicon cells at different E:T ratios (1:1, 1:5 and 1:10). After 24 hours, cells were transferred to FACS tubes and 4`,6-diamidino-2-phenylindole (DAPI) was added before analysis.

Procedure:

- Plate 5 x 10^4 NK cells twice, separately, in 96-well plates
- Stimulate cells with 25 U/ml IL-2 for 18 h
- Transfer stimulated NK cells to each 5 x 10^4 Huh7-HCVreplicon cells in 48-well plates and add 5 µl anti-CD107a-PE immediately
- Incubate for 60 min.
- After incubation add 1 µl Golgi-stopp and incubate for 4 h
- Transfer cell suspensions into FACS tubes
- Bring to a volume of 3 ml with PBS, and centrifuge for 10 min., 300*g, RT
- Discard supernatant and resuspend cells in 150 ml PBS
- Perform cell surface staining with 5 µl anti-CD56-APC, 1 µl anti-CD3-PerCP1 and 1 µl DAPI (20 sec. before measurement)
- Incubate in the dark for 20 min., RT
- Bring to a volume of 3 ml, with PBS and spin down for 10 min., 300*g, RT
- Discard supernatant and fix cells in Cellfix

- Analyse immediately or store cells in the dark at 4 °C, for 24 h max.

3.10 Cell culture / Human hepatoma 7 (Huh7) HCVreplicon cells

For a long time there was no suitable cell culture system to study hepatitis C virus replication. In our study we used the Huh7-HCVreplicon cell system. Replicon is a DNA or RNA sequence, which enables independent replication. Here, the HCV subgenomic replicon was constructed from viral RNA isolated from the liver of an infected individual. The replicon has a G418 (Neomycin) resistance and encodes also a luciferase reporter gene for the quantitation of hepatitis C virus replication. This cell culture system with replicating HCV-RNA allows for both, the inhibition of the virus replication as well as better research and understanding of the defense mechanisms of the effector cells [49], [50].

The Huh7-HCVreplicon cells were kindly provided by V. Lohmann and R. Bartenschlager (University of Heidelberg, Heidelberg, Germany). Cells were cultivated in an incubator at 37°C and 5% CO_2 and were grown in high-glucose Dulbecco's modified Eagle's medium (4.5 g/l) supplemented with glutamine, 10% fetal calf serum, 1% non-essential amino acids, and 1% penicillin/streptomycin. Blasticidin S hydro- chloride (3 lg/ml) and G418 (1 mg/ml) were added to cells containing subgenomic replicons. Huh7-HCVreplicon cells were passaged twice a week.

3.11 Antiviral Activity Assay (Luciferase)

Huh7-HCVreplicon cells were seeded in 12-well plates. After 3 hours, medium was removed and replicon cells were co-cultured with PBMC and purified NK- or NK-like T cells at different E:T ratios overnight in the absence or presence of IL-2 (25 U/ml) or IL-12 (1ng/ml) and IL-15 (10 ng/ml). In some experiments Huh7-HCVreplicon cells were cultivated for 24 hours with supernatants obtained from the corresponding direct co-culture experiments. The assay was performed using the Steady-Glo Luciferase Assay System and luminescence was measured with Tecan infinite M200. Part of the resulting supernatants from the co-culture was also collected for determination of aspartate aminotransferase (AST) levels as marker for cell damage.

3.12 Blocking Experiments with mAbs

In order to analyze a functional correlation of NKp46 and NKG2D regarding the activation of the NK cells via these receptors and the possibility of IFN-γ to block viral replication *in vitro* in Huh7-HCVreplicon cells, neutralizing antibodies were used to block these molecules during the effector: target co-incubation.

3.13 Statistical Analysis

The measured results were obtained on FACS Canto II and recorded as FCS files in a logarithmically scaled format. The evaluation was conducted with FlowJo Version 7.5.5. Statistical analysis was performed using the software GraphPad Prism Version 5.0a. Wilcoxon–Mann–Whitney test was used to compare NK-cell phenotype, cytotoxic activity, cytokine levels, and HCV replication activity. Unpaired t-tests were carried out in order to calculate the statistical significance. Correlations were analyzed using linear regression. A two-sided P value of less than 0.05 was considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

4 Results

4.1 Frequency and phenotype of NK cells in acute hepatitis C in HIV+ patients

At the beginning of our study we determined the influence of acute HCV infection on the NK cell pool composition as well as the phenotype of the cells. Here, we compared circulating NK cells from HCV acutely infected patients (HIV/aHCV) with HIV+ mono-infected patients (HIV), HIV+ patients with an established chronic HCV infection (HIV/cHCV) and healthy individuals.

Patients with acute HCV/HIV co-infection displayed the lowest frequency of all circulating NK cells compared to the control groups, whereas numbers of CD56bright NK cells were increased. However, statistical significance was found only with respect to healthy individuals (Fig. 1A).

Differences in NK cell populations did not reach statistical significance when patients with different outcomes of acute HCV infection were analyzed separately (Fig. 1B). Then, we studied the surface expression of differentiation/maturation markers on NK cells. Among all examined markers (CD57, CD62L, CD127 and CD161), we found that patients with a self-limited course of acute HCV infection expressed significantly higher numbers of CD62L in NK cells, both in the CD56bright and CD56Dim NK cell subpopulations, than patients who developed chronic infection (Fig. 1D). No other significant differences were observed regarding the other markers (Fig. 1C/E/F).

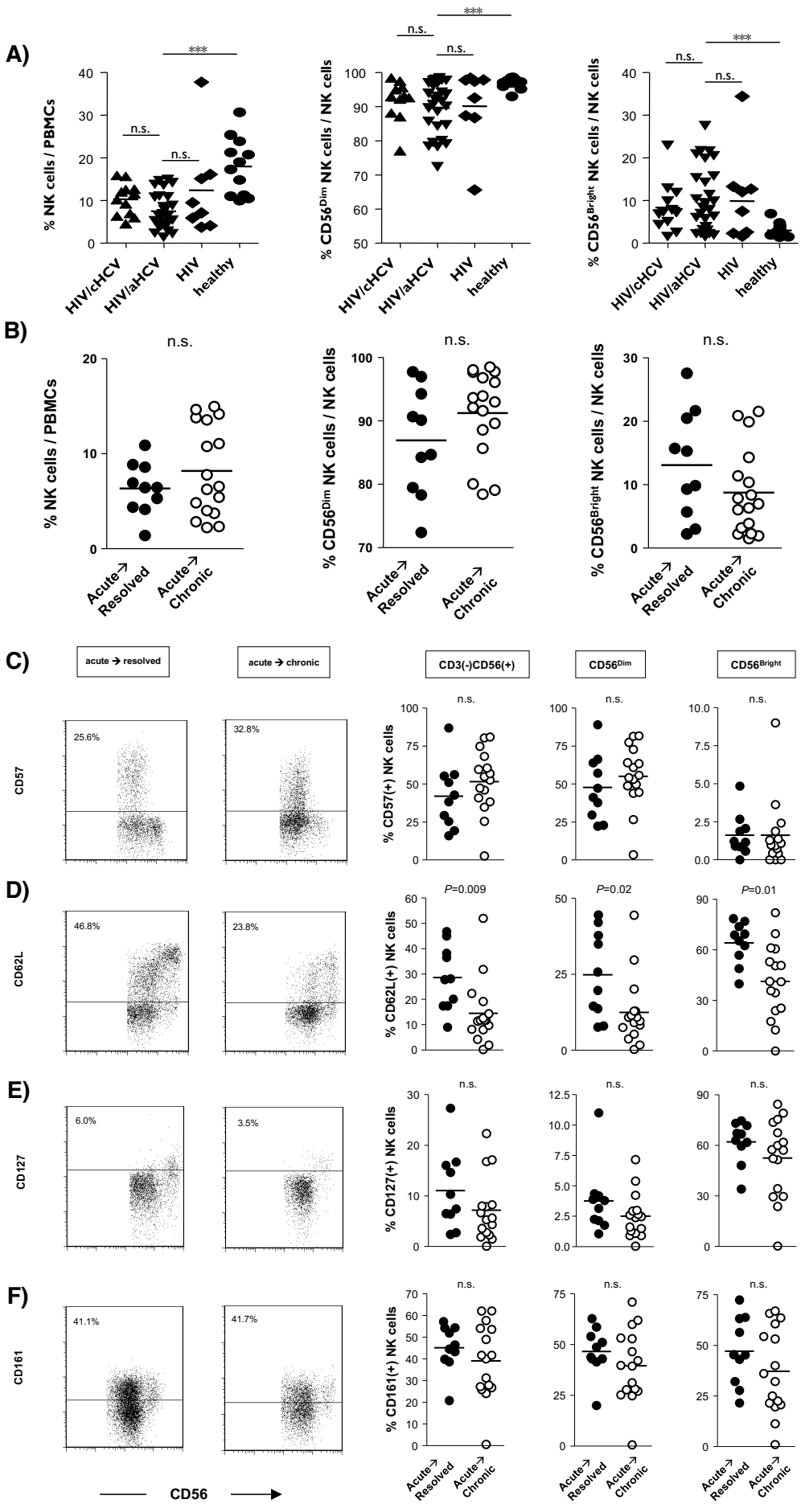
A)
% NK cells / PBMCs
% CD56Dim NK cells / NK cells
% CD56Bright NK cells / NK cells
n.s.
***
HIV/cHCV
HIV/aHCV
HIV
healthy
B)
Acute→ Resolved
Acute→ Chronic
C)
acute → resolved
acute → chronic
CD3(-)CD56(+)
CD56Dim
CD56Bright
CD57
% CD57(+) NK cells
25.6%
32.8%
D)
CD62L
% CD62L(+) NK cells
46.8%
23.8%
P=0.009
P=0.02
P=0.01
E)
CD127
% CD127(+) NK cells
6.0%
3.5%
F)
CD161
% CD161(+) NK cells
41.1%
41.7%
CD56

Fig. 1 Distribution of NK cells in acute hepatitis C in HIV+ patients. (A) Comparison of percentages of total peripheral NK cells (left panel), CD56Dim (middle panel) and CD56bright (right panel) NK cells in HIV+ patients with chronic (n=12) or acute (n=27) HCV, in HIV+ mono-infected patients (n=8) and healthy controls (n=12). (B) Comparison of the percentages of total peripheral NK cells (left panel), CD56Dim (middle panel) and CD56bright (right panel) NK cells in HIV+ patients with a self-limited (n=10) and chronic (n=17) course of HCV infection. (C/D/E/F) Frequency of total NK cells (left panel), CD56Dim (middle panel) and CD56bright (right panel) NK cells expressing CD57, CD62L, CD127 and CD161 respectively, in HIV+ patients with a self-limited (n=10) and chronic (n=16) course of HCV infection. *P≤0.05, **P≤0.01, ***P≤0.001

4.2 Peripheral NK cells are activated in acute hepatitis C/HIV co-infection

Our next step was to examine whether HCV infection was associated with altered activation levels of NK cells and their functional potency. Our analysis regarding the functional activity of NK cells revealed a significantly stronger degranulation in patients with acute hepatitis C compared to the control groups (Fig. 2A). Furthermore, NK cells from acutely infected patients showed the strongest expression of the activation marker CD69 (Fig.2B).

When patients with different outcomes of aHCV were analyzed separately we found weaker induction of CD107a (Fig. 2C) but higher expression of CD69 (Fig. 2D) in patients who subsequently cleared the infection.

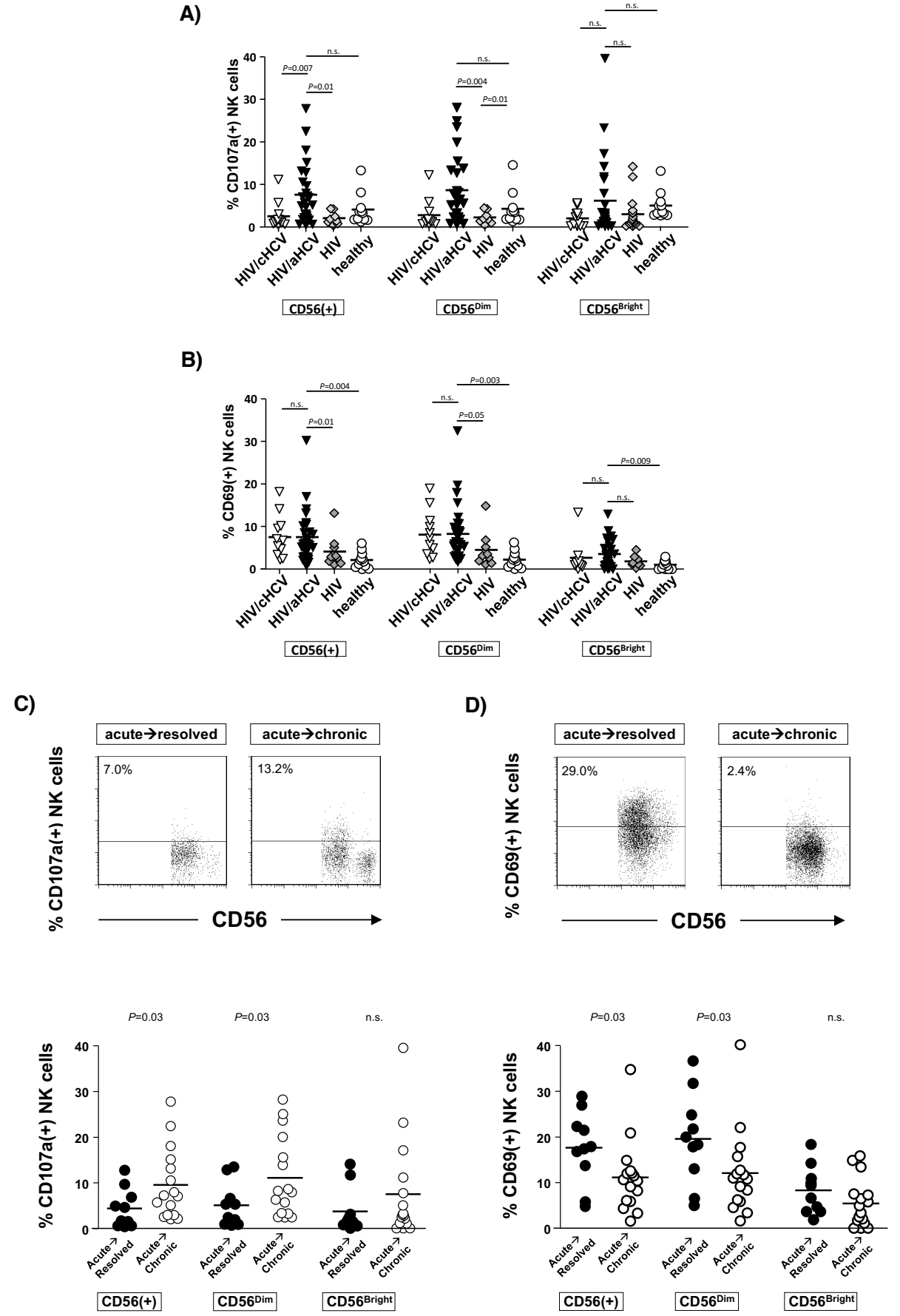
A)
% CD107a(+) NK cells
P=0.007
P=0.01
n.s.
P=0.004
P=0.01
n.s.
n.s.
n.s.
HIV/cHCV
HIV/aHCV
HIV
healthy
CD56(+)
CD56Dim
CD56Bright
B)
% CD69(+) NK cells
P=0.004
n.s.
P=0.01
P=0.003
n.s.
P=0.05
P=0.009
n.s.
n.s.
C)
acute→resolved
acute→chronic
7.0%
13.2%
% CD107a(+) NK cells
CD56
D)
29.0%
2.4%
% CD69(+) NK cells
P=0.03
P=0.03
n.s.
Acute→ Resolved
Acute→ Chronic

Fig. 2 Circulating NK cells are activated in acute hepatitis C/HIV co-infection. (A) Shows the percentage of degranulation (CD107a) after IL-2 stimulation as well as CD69 expression (B) on total NK cells, CD56Dim and CD56bright NK cells in HIV+ patients with chronic (n=11) or acute (n=27) HCV, in HIV mono-infected patients (n=9) and healthy individuals (n=12). (C) Compares the percentage of degranulation (CD107a) as well as CD69 expression (D) on total NK cells, CD56Dim and CD56bright in patients with a self-limited (closed circles) and chronic course (open circles) of acute HCV infection in HIV+ patients.

4.3 Spontaneous clearance of acute hepatitis C is dependent on robust anti-HCV NK cell activity

In order to clarify if there were any differences in NK cell functions with respect to the outcome of the hepatitis C infection we co-incubated IL—2-stimulated NK cells with Huh7-HCVreplicon cells. We found a significantly higher frequency of degranulating CD56bright NK cells (Fig. 3A) as well as a significantly higher efficacy of NK cells in inhibiting HCV replication *in vitro* (Fig. 3B/C) in patients with self-limited hepatitis C infection than in patients who developed a chronic course of infection.

Inhibition of HCV replication was not correlated to the extent of cytolytic activity of NK cells, because we could not observe any correlation between degranulation and inhibition of HCV replication in the replicon cells (Fig. 3D).

In line with this findings, we did also not find, even at high 1:1 E:T ratio, any relationship between inhibition of HCV replication and NK cell cytotoxicity, when we analyzed specific lysis in the killing assays and AST levels, respectively (Fig. 3E/F). These observations suggest that non-cytolytic mechanisms could play a more important role in anti-HCV NK cell activity.

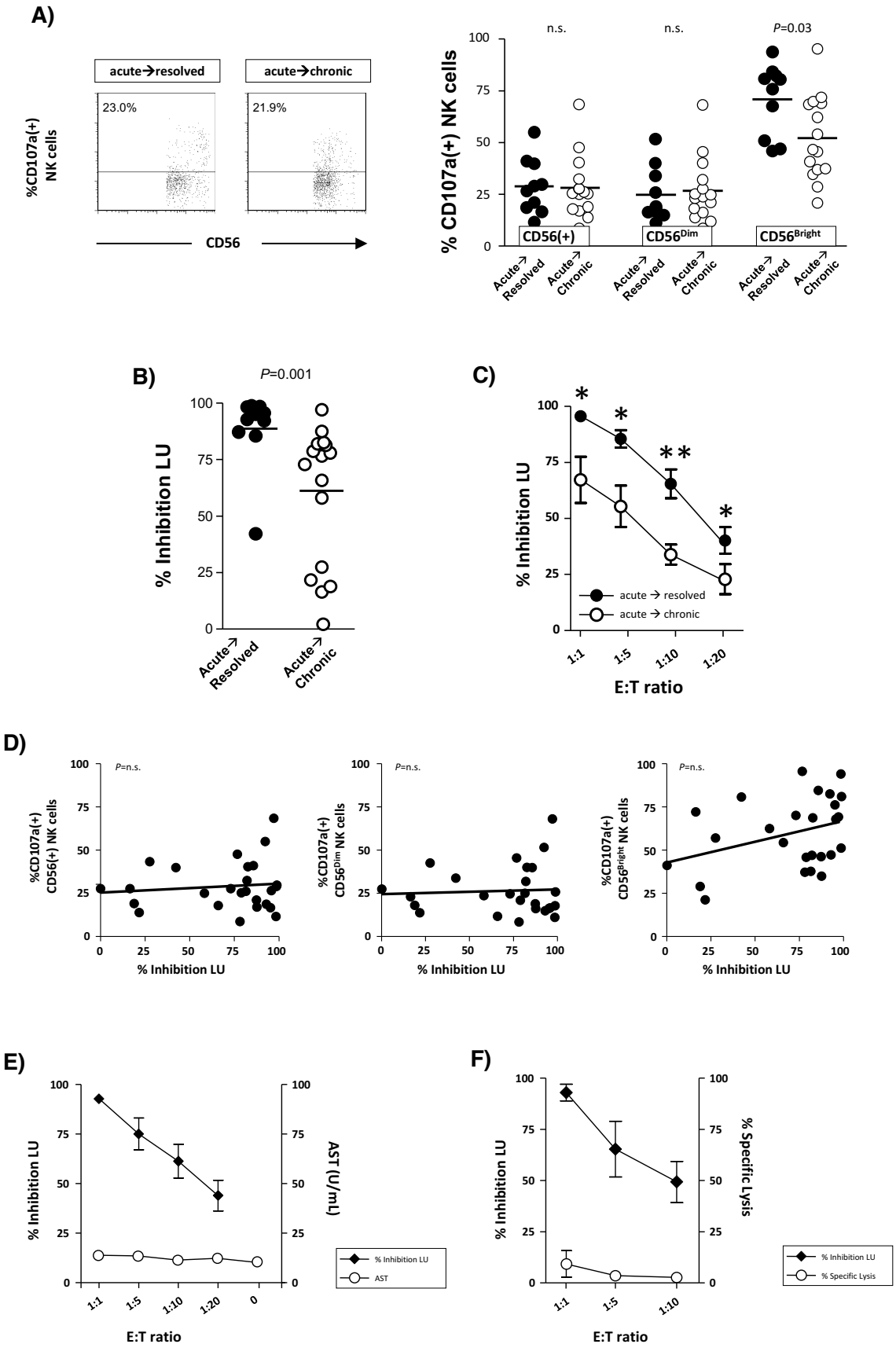
A)
acute→resolved
acute→chronic
23.0%
21.9%
%CD107a(+) NK cells
CD56
n.s.
n.s.
P=0.03
% CD107a(+) NK cells
CD56(+)
CD56Dim
CD56Bright
Acute→ Resolved
Acute→ Chronic
B)
P=0.001
% Inhibition LU
C)
% Inhibition LU
acute → resolved
acute → chronic
E:T ratio
D)
P=n.s.
%CD107a(+) CD56(+) NK cells
%CD107a(+) CD56Dim NK cells
%CD107a(+) CD56Bright NK cells
% Inhibition LU
E)
% Inhibition LU
AST (U/mL)
E:T ratio
F)
% Specific Lysis

Fig. 3 Spontaneous clearance of acute HCV infection in HIV+ patients is associated with an effective NK cell-mediated inhibition of HCV replication. (A) Shows degranulation rates of IL-2-stimulated NK cells acquired from patients with a self-limited (n=10) and chronic course (n=15) of acute HCV infection after co-incubation with Huh7-HCVreplicon cells. (B) Inhibition of luciferase activity (LU) after co-incubation of Huh7-HCVreplicon cells with NK cells from patients with a self-limited (n=10) and chronic course (n=16) of acute HCV infection. (C) Comparison of inhibition of luciferase activity (LU) after co-incubation of Huh7-HCVreplicon cells with NK cells from patients with a self-limited and chronic course of acute HCV infection at different E:T ratios. (D) Correlation of CD107a+ NK cells (left panel), CD56Dim (middle panel) and CD56bright (right panel) NK cells and NK cell mediated inhibition of HCV replication in replicon cells. (E) Shows the AST levels in the supernatants of the NK cell / Huh7-HCVreplicon cells co-culture at different E:T ratios contrasted to inhibition of HCV replication. (F) NK cell-mediated specific lysis of Huh7-HCVreplicon cells at different E:T ratios contrasted to inhibition of HCV replication. *P≤0.05, **P≤0.01, ***P≤0.001

4.4 Spontaneous clearance of acute hepatitis C in HIV+ patients is associated with a strong IFN-γ mediated anti-HCV activity of NK cells

As result of the previous findings we then studied whether soluble factors are responsible for NK cell-mediated inhibition of HCV replication favoring cure of acute hepatitis C. Therefore, we analyzed IFN-γ since this cytokine has strong antiviral activity.

First, we analyzed the potential of NK cell capacity to produce IFN-γ after IL-12/IL-15 stimulation and we found significantly higher numbers of CD56bright NK cells with IFN-γ secretion from patients who spontaneously cleared the infection than in patients with a chronic course of infection (Fig. 4A).

Moreover, we found a positive correlation between IFN-γ secretion in NK cells and inhibition of replication in the HCV replicons, supporting a role of this cytokine in anti-HCV NK cell activity (Fig. 4B).

In order to validate these observations, we performed further experiments. In line with the prior results we studied IFN-γ concentrations in supernatants after co-incubation of NK cells with Huh7-HCVreplicon cells. We found significantly higher concentrations of IFN-γ in the supernatants from patients who spontaneously cleared HCV infection compared to patients developing chronic HCV infection (Fig. 4C). Consequently, supernatants of NK cells from patients with a self-limiting course of HCV infection showed significantly greater ability to inhibit viral replication than supernatants from patients who

progressed to chronic hepatitis C infection (Fig. 4D). Additionally, *in vitro* anti-HCV activity of NK cells from patients who cleared hepatitis C virus naturally was significantly diminished after co-incubation with a specific IFN-γ antibody and decreased to the same level of inhibition of HCV replication with patients who progressed towards chronic infection (Fig. 4E).

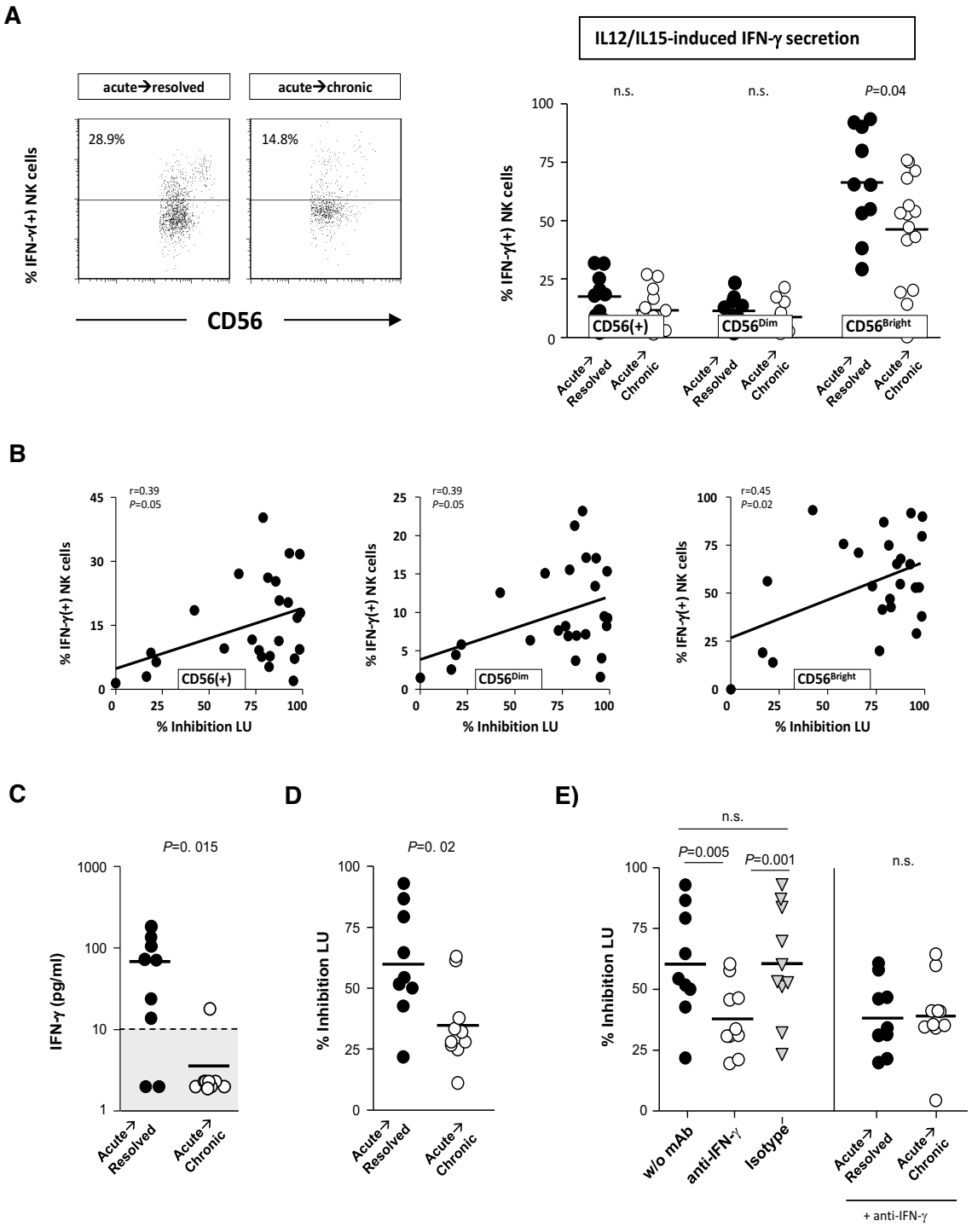

Fig. 4 IFN-γ is critically involved in NK cell-mediated inhibition of HCV replication. (A) NK cells from patients with a self-limited (n=10) and chronic course (n=15) of acute hepatitis C infection were stimulated with IL-12 and IL-15 in order to assess their capacity to secrete IFN-γ. (B) Correlation of cytokine induced IFN-γ secretion of total NK cells (left panel), CD56Dim (middle panel) and CD56bright (right panel) NK cells and inhibition of HCV replication (LU). (C) After co-incubation of NK cells from patients with different outcomes of acute HCV infection in follow up (self-limited n=9 and chronic course n=10) with Huh7-HCVreplicon cells, supernatants were collected and tested for IFN-γ release by ELISA. (D) Following co-incubation of NK cells with Huh7-HCVreplicon cells, supernatants were collected and tested for their ability to block HCV replication (self-limited n=9 and chronic course n=10). (E) Illustrates the distribution of the effect of IFN-γ blockade on NK cell-mediated anti-HCV activity in patients with a self-limited course (n=10) of acute HCV infection (left graph) and the impact of a specific IFN-γ neutralizing antibody on anti-HCV activity of NK cells obtained from patients with a self-limited (n=9) and chronic course (n=10) of acute hepatitis C (right graph). The percentage of inhibition LU was calculated using cross-multiplication between samples and controls *P≤0.05, **P≤0.01, ***P≤0.001

4.5 Receptors NKG2A, NKG2C and CD158e are not involved in anti-HCV activity of NK cells

The activity of NK cells is regulated by activating as well as inhibiting receptors. In order to elucidate whether the ability to clear the HCV infection in its early stage is related to the expression of different NK cell receptors we analyzed the surface expression of NK cells. We found that NK cells from patients with a self-limited course of acute hepatitis C expressed significantly higher levels of NKG2A, while expression of NKG2C and CD158e was reduced when compared with patients with a chronic course of AHC infection (Fig. 5). Further statistical analysis showed no correlation between expression of CD158e and NKG2A, and anti-HCV activity of NK cells (Fig. 5A/B right graphs). The frequency of NKG2C was inversely correlated with anti-HCV NK cell functions like IFN-γ secretion and *in vitro* inhibition of HCV replication (Fig. 5C), implying that these receptors are not involved in antiviral activity against HCV.

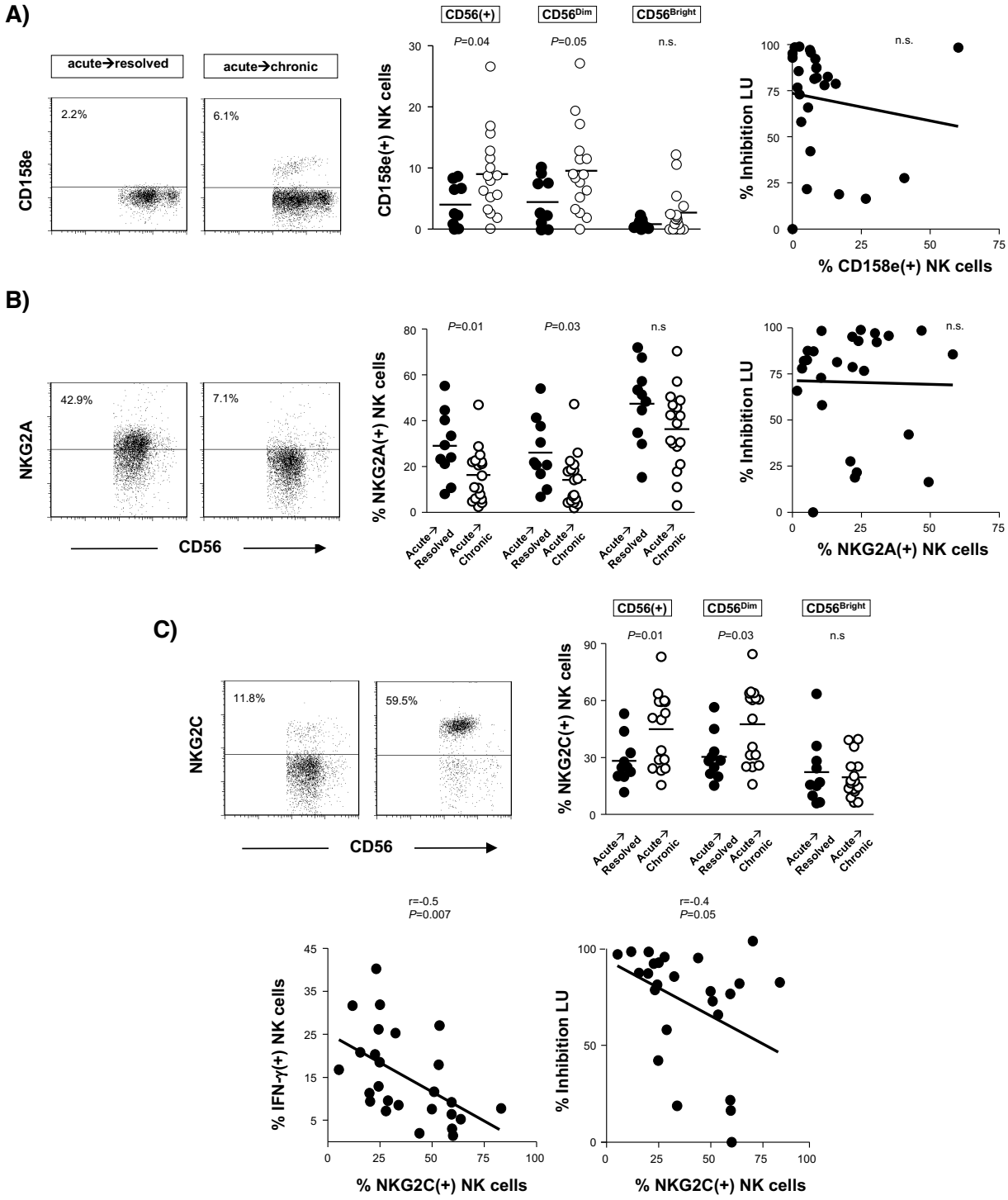

Fig. 5. Expression of CD158e, NKG2A and NKG2C in aHCV/HIV co-infection. Comparison of CD158e, NKG2A and NKG2C expression on NK cells respectively, in patients with a self-limited and chronic course of acute hepatitis C virus infection. Correlation of frequency of these markers with NK cell anti-HCV activity (A/B/C) as well as correlation of the frequency of NKG2C+ NK cells with the proportion of IFN-γ+ NK cells (C).

4.6 NKp46 and NKG2D regulate antiviral NK cell activity and may influence outcome of acute hepatitis C infection

Unlike the hitherto characterized NKRs, we observed a direct role of NKp46 and NKG2D strengthening the inhibition of HCV replication in the replicon cells. We found expression of both activating NK cell receptors NKp46 and NKG2D to be expressed in significantly higher quantities on NK cells from patients who naturally cleared the infection compared to patients who subsequently developed chronic hepatitis C infection (Fig. 6A). Of note, the expression of both receptors was positively correlated with IFN-γ production as well as the extent of NK cell-mediated blocking of HCV replication *in vitro,* underlining an important role for anti-HCV NK cell activity (Fig. 6B). Lastly, the functional roles of NKp46 and NKG2D were confirmed by blocking experiments. After blocking NKp46 and NKG2D with specific mAbs we observed significantly lower NK cell-mediated inhibition of HCV replication *in vitro* (Fig. 6C).

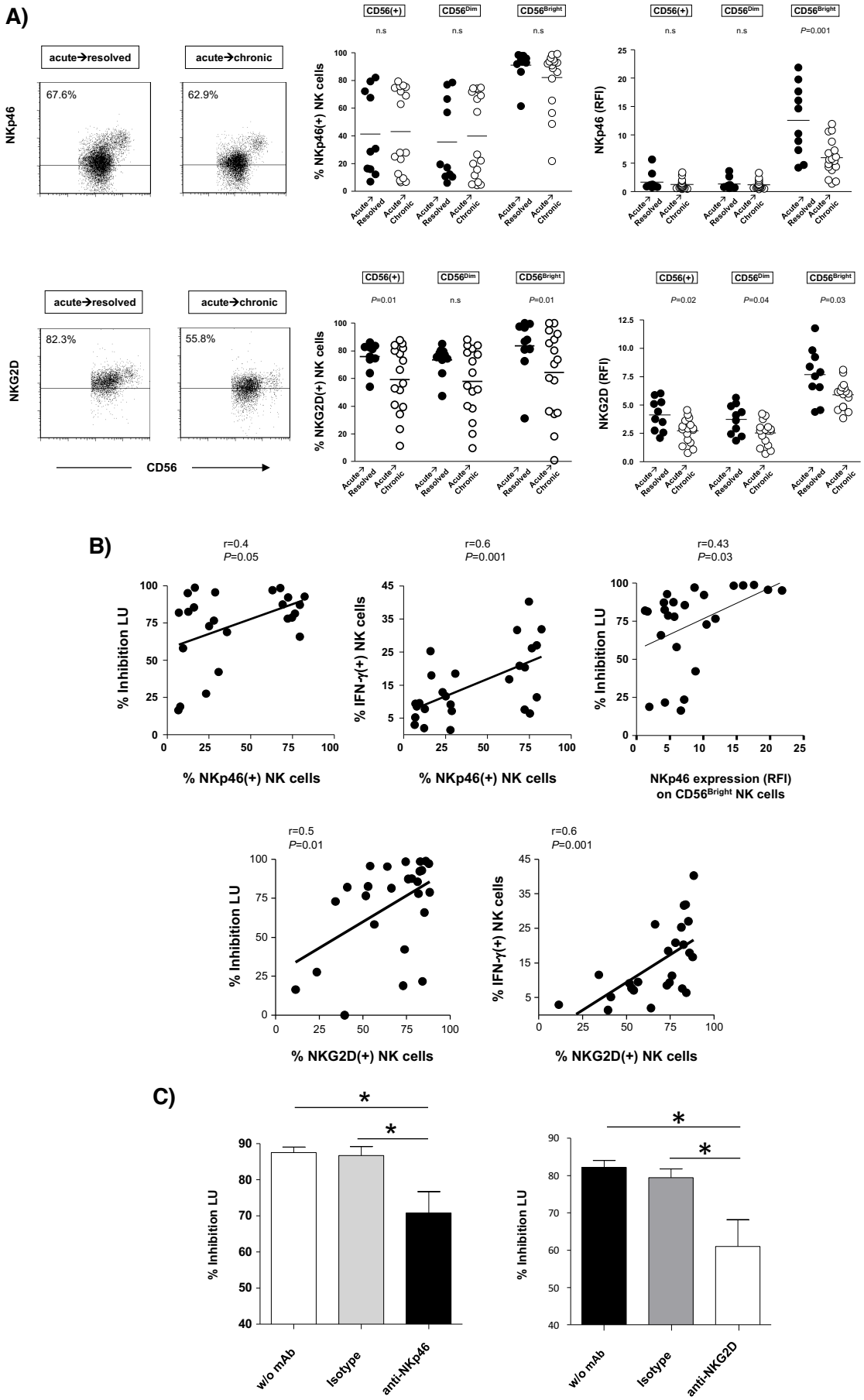
A)
acute→resolved
acute→chronic
NKp46
67.6%
62.9%
NKG2D
82.3%
55.8%
CD56
CD56(+)
CD56Dim
CD56Bright
n.s
P=0.001
P=0.01
P=0.02
P=0.04
P=0.03
% NKp46(+) NK cells
NKp46 (RFI)
% NKG2D(+) NK cells
NKG2D (RFI)
Acute→ Resolved
Acute→ Chronic
B)
r=0.4
P=0.05
r=0.6
P=0.001
r=0.43
P=0.03
% Inhibition LU
% IFN-γ(+) NK cells
% NKp46(+) NK cells
NKp46 expression (RFI) on CD56Bright NK cells
r=0.5
P=0.01
r=0.6
P=0.001
% NKG2D(+) NK cells
C)
*
% Inhibition LU
w/o mAb
Isotype
anti-NKp46
anti-NKG2D

Fig. 6. NKp46 and NKG2D are involved in anti-HCV activity of NK cells in acute HCV/HIV co-infection. (A) Frequency and density (RFI) of NKp46 and NKG2D surface expression on NK cells from patients with a self-limited and chronic course of acute HCV/HIV co-infection. (B) Correlation between frequency and density of NKp46 / NKG2D and inhibition of HCV replication or proportion of IFN-γ+ NK cells in HIV+ patients with an acute hepatitis C virus infection. (C) Effect of blockade of NKp46 and NKG2D receptors with specific neutralizing antibodies. *P≤0.05, **P≤0.01, ***P≤0.001

4.7 Frequency and phenotype of NK-like T cells in acute hepatitis C in HIV+ patients

Next, we analyzed the influence of acute HCV infection on the NK-like T cell pool as well as the phenotype of the NKT-cells. Here, we compared circulating NK-like T cells from HCV acutely infected patients (HIV/aHCV) with HIV+ mono-infected patients (HIV), HIV+ patients with an established chronic HCV infection (HIV/cHCV) and healthy individuals. We did not observe any significant differences in the NK-like T cell pool among our study groups (Fig. 7A). However, we found significantly higher percentages of CD158b and NKG2A expression on NK-like T cells in patients with HCV infection than in the control groups (Fig. 7B). We did not observe any other differences regarding the expression of NKG2C and NKG2D between the studied groups (Data not shown). Of note, when NK-like T cells from patients with different outcomes of acute hepatitis C infection were analyzed separately, we found self-limiting course to be associated with a significant lower expression of NKG2C (Fig. 7C).

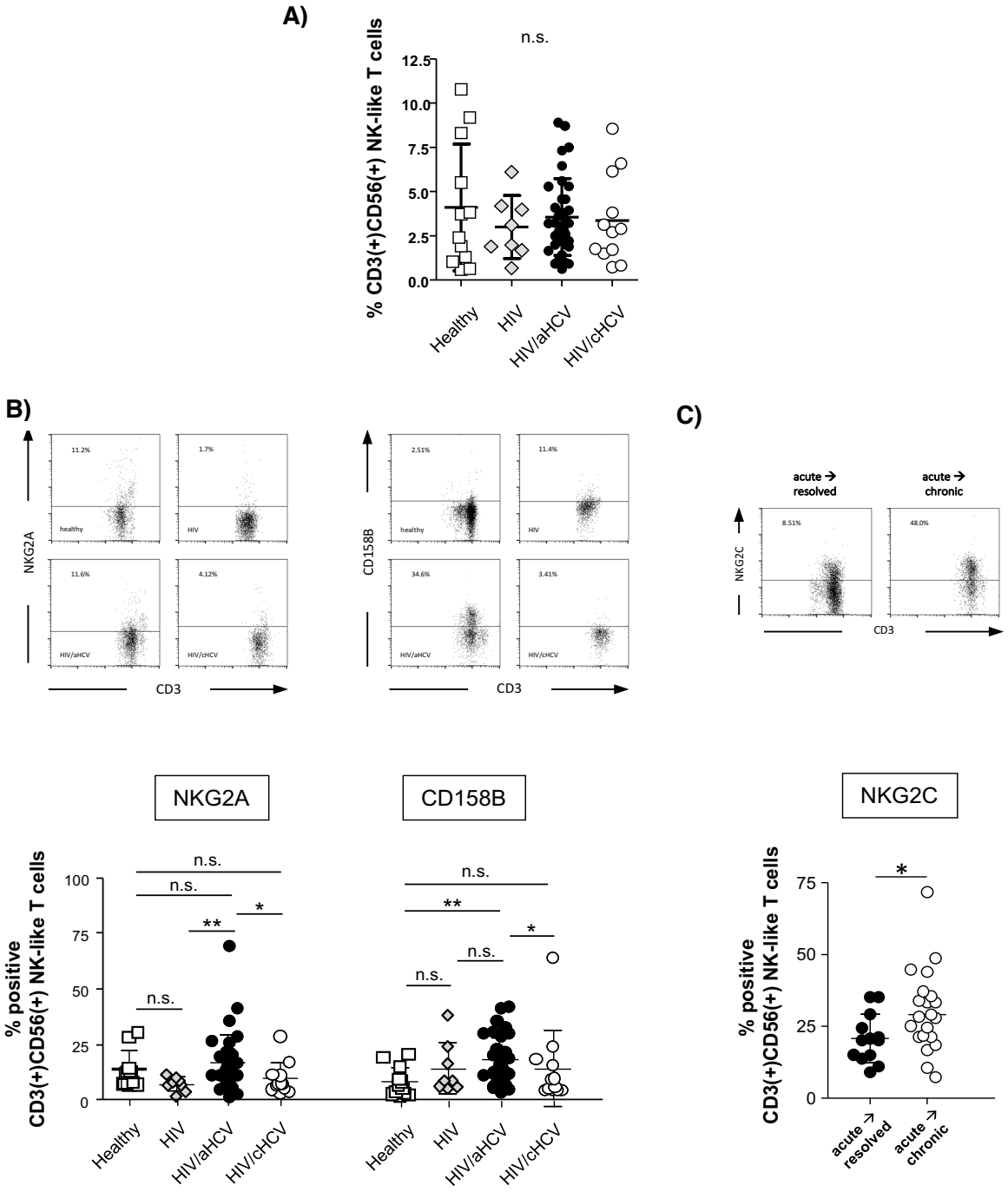

Fig. 7 NK-like T cell frequency as well as expression of C-type lectin and KIR receptors in peripheral blood in HIV+ patients with acute hepatitis C. (A) Frequency of peripheral NK-like T cells in healthy controls (n=12), in HIV mono-infected patients (n=8) and HIV+ patients with acute (n=36) or chronic (n=12) hepatitis C infection. (B) Percentage of NKG2A+ and CD158b+ NK-like T Cells in healthy controls (n=12), in HIV mono-infected patients (n=8) and HIV+ patients with acute (n=36) or chronic (n=12) hepatitis C infection. (C) Percentage of NKG2C+ NK-like T cells in HIV+ patients with a self-limited (n=13) and chronic course (n=21) of acute hepatitis C virus infection. *P≤0.05, **P≤0.01, ***P≤0.001

4.8 Expression of maturation markers on NK-like T cells in acute HCV/HIV co-infection

The analysis of the maturation/differentiation markers revealed that acute hepatitis C in HIV+ patients is associated with a significantly reduced fraction of $CD161^{+}$ NK-like T cells compared to the other control groups. In contrast, NK-like T cells from patients with HIV infection expressed significantly lower CD27 and CD127 irrespective of HCV co-infection (Fig. 8). Regarding the expression of these markers in patients with different outcomes of acute hepatitis C infection, we could not observe any significant changes (Data not shown).

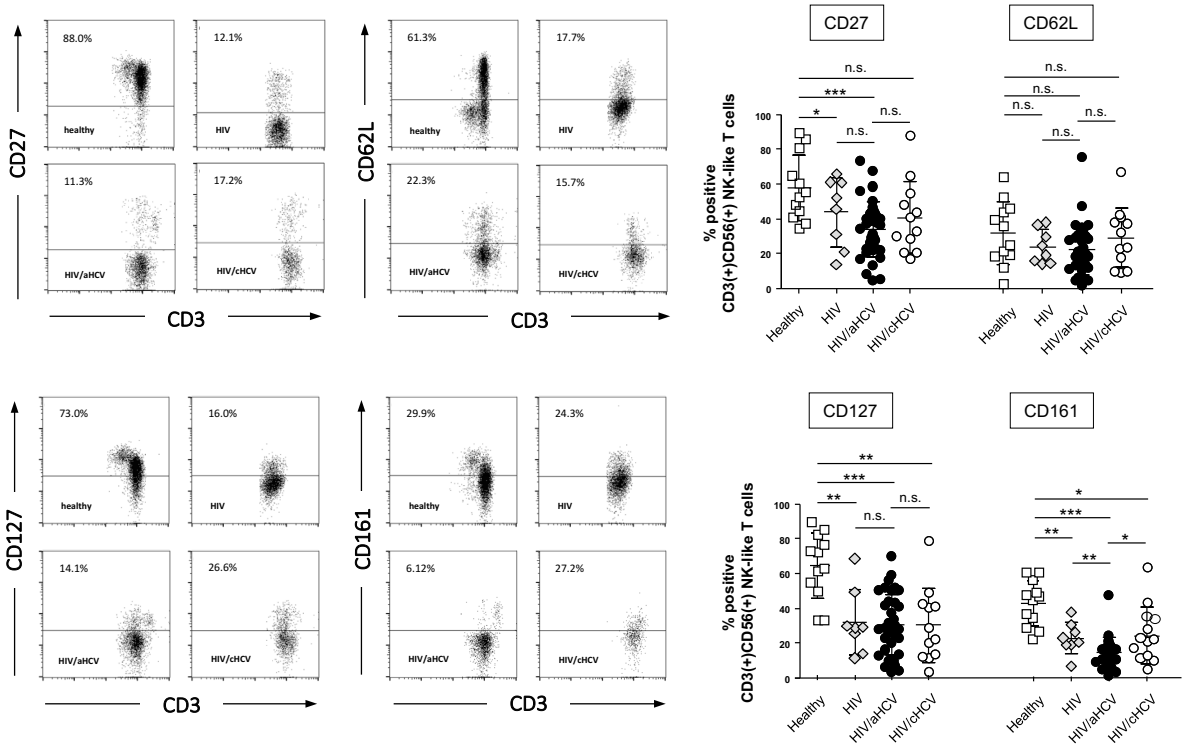

Fig. 8 Expression of maturation markers on CD3(+)CD56(+) NK-like T cells. Graph shows surface expression of the maturation/differentiation markers CD27, CD62L, CD127 and CD161 on NK-like T cells from healthy controls(n=12), HIV+ mono-infected patients (n=8) and HIV+ patients with acute (n=36) or chronic (n=12) hepatitis C infection. *P≤0.05, **P≤0.01, ***P≤0.001

4.9 Peripheral NK-like T cells are activated in acute hepatitis C/HIV co-infection

Our next step was to clarify whether HCV infection was associated with the activation level of NK-like T cells in acute HCV/HIV co-infection. Since the number of available lymphocytes was too low to isolate NK-like T cells from the patients, we performed the experiments with PBMCs. Our analysis regarding the cytotoxic activity of NK-like T cells revealed a significantly stronger degranulation (CD107a) in patients with acute hepatitis C compared to HIV mono-infected as well as HIV+ patients with chronic hepatitis C infection. When patients with different outcomes of acute HCV were analyzed separately we did not observe any differences (Fig. 9A). Furthermore, NK-like T cells from acutely infected patients showed the highest expression of the activation marker CD69 when compared to healthy individuals and HIV mono-infected patients. Moreover, we found patients with a self-limited course of HCV infection to display significantly higher expression of CD69 than patients with a chronic course of infection (Fig.9B).

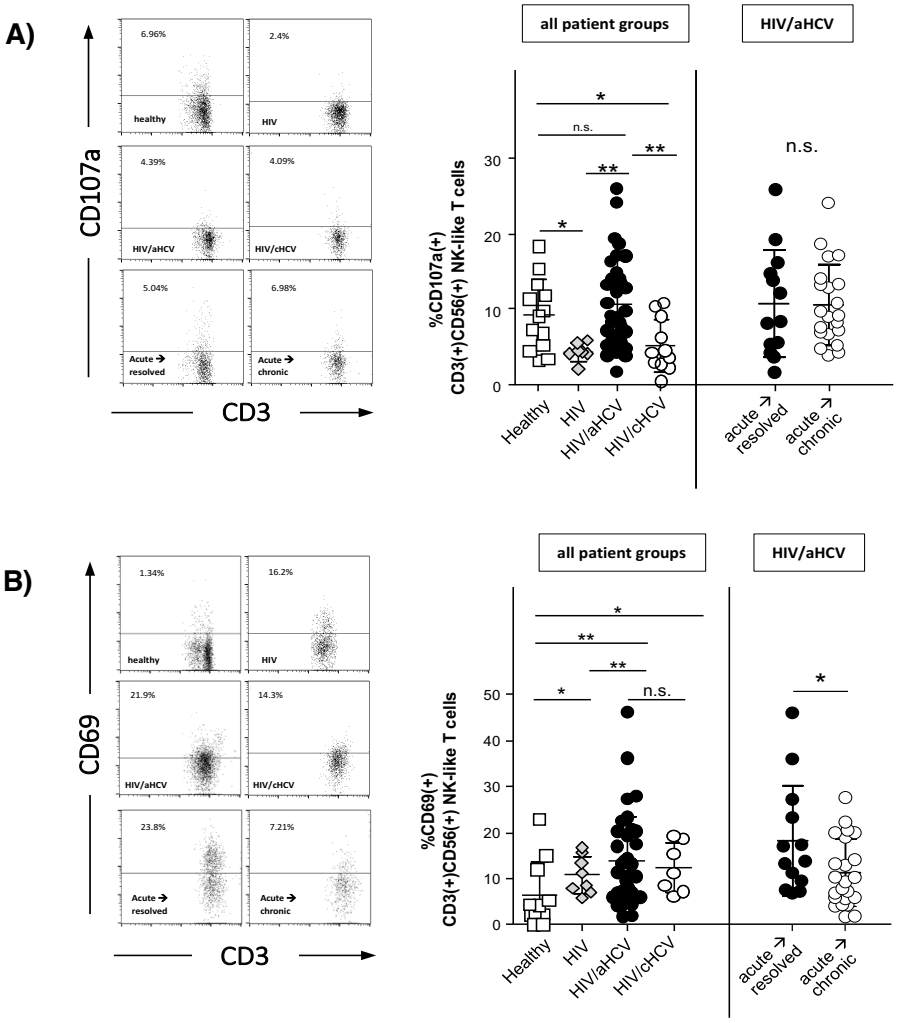

Fig. 9 Degranulation (CD107a) and activation level (CD69) of NK-like T cells in HIV+ patients with acute hepatitis C infection. (A) Degranulation of circulating NK-like T cells after co-incubation with Huh7-HCVreplicon cells in healthy controls (n=12), HIV+ mono-infected patients (n=8) and HIV+ patients with acute (n=36) or chronic (n=11) hepatitis C (left graph) and in HIV+ patients with a self-limited (n=13) or chronic (n=23) course of acute hepatitis C infection (right graph). (B) Activation level as defined by CD69 expression of peripheral NK-like T cells in healthy controls (n=12), HIV+ mono-infected patients (n=8) and HIV+ patients with acute (n=36) or chronic (n=7) hepatitis C (left graph) and in HIV+ patients with a self-limited (n=13) or chronic (n=23) course of acute hepatitis C infection (right graph). *P≤0.05, **P≤0.01, ***P≤0.001

4.10 HIV infection impairs the ability of CD3(+)CD56(+) NK-like T cells to inhibit HCV replication

Despite the fact that we did not find phenotypic alterations apart from upregulated expression of CD69 to be associated with spontaneous clearance, we also analyzed functions of CD3(+)CD56(+) NK-like T cells and their capacity to inhibit HCV replication. We found that unstimulated CD3(+)CD56(+) NK-like T cells from healthy donors displayed moderate inhibition of HCV replication, whereas IL-12/IL-15 stimulation significantly enhanced their antiviral capacity (Fig. 10A).

To elucidate whether CD3(+)CD56(+) NK-like T cells-mediated inhibition of HCV replication was cell-cell contact dependent we performed further experiments with the supernatants from the corresponding co-incubation experiments of CD3(+)CD56(+) NK-like T cells x Huh7-HCVreplicon cells. We found that supernatants from IL-12/IL-15 stimulated cells inhibited viral replication significantly more effectively than supernatants from resting NK-like T cells suggesting that non-cytolytic factors are involved in this mechanism (Fig. 10B). In support of this observation, we found only a negligible killing-activity (specific lysis, AST levels) of CD3(+)CD56(+) NK-like T cells after co-incubation with Huh7-HCVreplicon cells (Fig. 10C) but observed significantly increased production of IFN-γ after IL-12/IL-15 stimulation (Fig. 10D). We also found that CD3(+)CD56(+) NK-like T cell-dependent inhibition of HCV replication in HCV replicon cells as well as IFN-γ secretion were reduced after neutralization with a specific IFN-γ antibody (Fig. 10E). Finally, IL-12/IL-15 stimulated CD3(+)CD56(+) NK-like T cells from HIV infected patients showed significantly reduced efficacy inhibiting HCV replication *in vitro* as well as IFN-γ production when compared with cells from healthy controls (Fig. 10F/G).

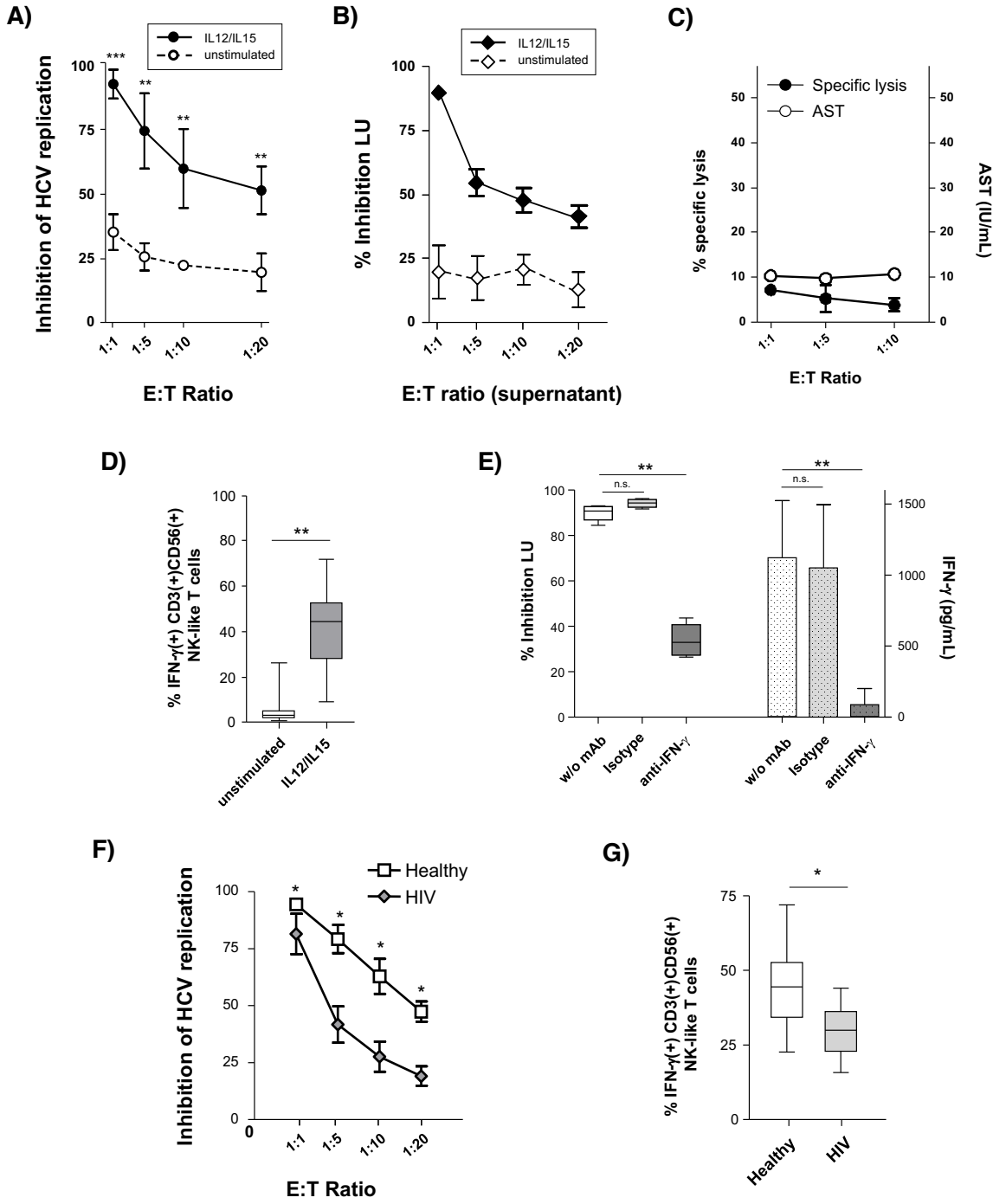

Fig. 10 The anti-HCV activity of NK-like T cells is impaired in HIV+ patients. (A) Unstimulated or IL-12/IL-15 pre-stimulated, isolated NK-like T cells from healthy donors (n=7) were co-incubated with Huh7-HCVreplicon cells over night at different E:T ratios in order to analyze their anti-HCV ability. (B) Supernatants from co-incubation of NK-like T cells / Huh7-HCVreplicon cells were analyzed with respect to inhibition of HCV replication (n=7). (C) NK-like T cell-mediated cytotoxicity and AST levels in supernatants were

measured after co-incubation of NK-like T cells with Huh7-HCVreplicon cells. (D) Unstimulated or IL-12/IL-15 pre-stimulated NK-like T cells from healthy donors (n=7) were tested for IFN-γ production. (E) Distribution of the effect of IFN-γ blockade on NK-like T cell-mediated anti-HCV activity in healthy donors (n=4). (F) NK-like T cells from healthy donors (n=7) and HIV+ patients (n=7) were stimulated with IL-12/IL-15 and then co-incubated with Huh7-HCVreplicon cells to analyze their ability to block HCV replication. (G) NK-like T cells from healthy donors (n=12) and HIV+ patients (n=8) were stimulated with IL-12/IL-15 and then tested for IFN-γ production. *P≤0.05, **P≤0.01, ***P≤0.001

4.11 CD3(+)CD56(+) NK-like T cells from HIV-positive patients with acute HCV infection display reduced IFN-γ Production

In order to clarify whether CD3(+)CD56(+) NK-like T cells play a decisive role concerning outcomes of acute hepatitis C in HIV infected patients, we analyzed their IFN-γ production. As shown in Fig. 11, IL-12/IL-15 stimulated CD3(+)CD56(+) NK-like T cells in acute HCV/HIV infection were associated with significantly lower IFN-γ production compared to all other control groups. Importantly, we did not find any significant differences in IFN-γ production between patients who spontaneously cleared their infection vs. patients who developed chronic HCV infection when isolated CD3(+)CD56(+) NK-like T cells were analyzed separately.

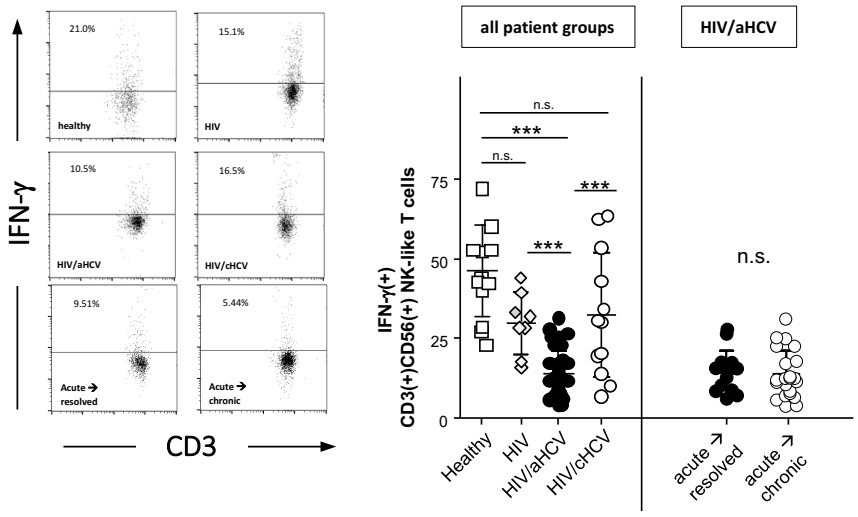

Fig. 11 IFN-γ production of NK-like T cells in HIV+ patients with acute hepatitis C. NK-like T cells from healthy donors (n=12), HIV+ mono-infected patients (n=8) and HIV+ patients with acute (n=36) or chronic (n=12) hepatitis C infection (left graph) as well as HIV+ patients with self-limited (n=13) or chronic (n=23) course of hepatitis C infection (right graph) were stimulated with IL-12/IL-15 and then analyzed with respect to IFN-γ production. *P≤0.05, **P≤0.01, ***P≤0.001

5 Discussion

Despite the pre-existing HIV infection, a small proportion of acute HCV infected patients can spontaneously clear their hepatitis C virus infection during the acute phase and completely eliminate the virus spontaneously. Until now, the mechanisms that favor spontaneous (natural) clearance are not understood sufficiently. Numerous studies indicated an important role for NK cells during hepatitis C infection, and recent analysis from several groups suggested distinct subpopulations to be involved in the early stage and determining outcomes of HCV infection [29], [30], [34]–[36], [51]–[55] . In our work we focused on frequency, phenotypes and functional alterations of NK and NKT cells and correlated them to spontaneous clearance of the acute HCV infection in HIV-positive patients. It should be noted that we are the only group that investigated the role of NK and NKT cells in acute hepatitis C / HIV co-infection. All previous studies were conducted on acute HCV mono-infection [29], [35], [36]. First, we wanted to analyze whether HCV infection has an impact on the relative abundance and distribution of the NK cells and their subsets. At this point, we found a decline on CD56Dim NK cells in acute hepatitis C when compared to healthy controls, which was in line with the results of other groups. While Pelletier et. al. and Alter et. al did not find any differences in the CD56Bright NK cell fraction, Amadei and our group, observed increased numbers of CD56Bright NK cells in resolvers when compared to healthy donors [29], [35], [36]. The total numbers of NK cells in acute hepatitis C varied also between the different studies, while Amadei et. al. and our group found a depletion in aHCV infection which was in contrast to the findings of Alter et. al. [29], [35]. Pelletier et al. suggested that these differences in the composition of the NK cell pool might be an effect of a persistent stimulation of the CD56Dim cells or a switch to activated CD56Bright NK cells [36]. In contrast, other studies on NK cells showed that it is more likely that CD56Bright cells show CD56Dim NK cells features after stimulation [56]. Consequently, the role of NK cells in acute hepatitis C is still discussed controversially owing to the divergent data. Therefore, more studies on the frequency and composition of NK cells are needed to understand differences in recruitment between peripheral NK cells and liver resistant NK cells in hepatitis C infection. Despite the fact that the frequency of NK cells in peripheral blood may reveal only vague information, the functional properties of these cells are considered to be pivotal for the spontaneous cure of acute hepatitis C in HIV+ patients.

Although all previous studies in acutely HCV mono infected patients showed also significant differences in phenotype and activity of NK cells, they did not find any correlations between NK cell functions and outcomes of acute HCV infection [29], [35], [36]. Recently, we and others have already shown that non-cytotoxic functions of NK cells as well as HCV-specific CD8+ T cells are critically involved in the control of chronic hepatitis C infection [34], [57]. Here we provided first evidence showing that NK cells are critically involved in outcome of acute HCV infection favoring spontaneous clearance by blocking the hepatitis C virus replication. We present a cascade of evidence suggesting IFN-γ secretion of NK cells to be a key parameter for the outcome of acute HCV infection. We show IL-12/IL-15-induced IFN-γ secretion to be significantly higher in CD56bright NK cells from resolvers compared to chronic patients. The frequency of IFN-γ+ NK cells was positively correlated with *in vitro* anti-HCV activity of NK cells against HCV replicon cells while NK cell *in vitro* degranulation (CD107a) did not reveal any correlation between NK cell cytolytic activity and inhibition of HCV replication. In line with this, we demonstrated a significantly higher concentration of IFN-γ in supernatants from patients who spontaneously resolved HCV infection after co-incubation with Huh7-HCVreplicon cells compared to supernatants from patients who developed a chronic course of infection. Furthermore, supernatants from resolvers showed a significantly higher ability to inhibit HCV replication *in vitro* and neutralization with a specific anti-IFN-γ antibody inhibited this phenomenon compared to patients who subsequently developed chronic infection.

Taken together, these findings indicate a key role for IFN-γ secretion from NK cells in anti-HCV activity, suggesting a possible mechanism which supports spontaneous clearance of infection in the early acute phase. Our results correspond with observations made in CD8+ T cells during HCV mono infection where IFN-γ production in liver was correlated with decreased HCV RNA levels [58]. In addition, accumulation of HCV specific IFN-γ producing CD8+ T cells in livers of patients without significant disease further supports a non-cytolytic pathway in the acute phase of the infection [59], [60]. Nevertheless, all other groups who worked on acute HCV infection could not find any significant differences between IFN-γ capacities of NK cells and outcomes of HCV infection. Amadei et. al. and Alter et. al. even showed that CD56Dim NK cells tended to exert more IFN-γ compared to their counter partners CD56Bright NK cells. Pelletier et. al. did not observe any changes at all [29], [35], [36].

These differences between our results and those of the other groups could be due to contrasting stimulations of the NK cells. Pelletier et. al. and Alter et. al. stimulated the NK cells with K562 and 221 target cells without prior cytokine stimulation, while Amadei et. al. used IL-12 as stimulator in their experiments and we used both cytokines IL-12 and IL-15 to achieve maximum stimulation. In our experiments, patients with acute HCV infection could also secrete higher amounts of IFN-γ compared to patients in Amadei et. al., an observation which may be linked to the long-term activation by the persisting HIV infection in our cohort. However, according to our results stimulation with cytokines seems to be the better way to analyze the capacity of NK cells to produce IFN-γ.

Next, our laboratory analysis of blood revealed significantly higher alanine transaminase (ALT) and aspartate transaminase (AST) levels in patients with acute hepatitis C than in patients with chronic hepatitis C infection. However, our specific *in vitro* lysis experiments regarding cytotoxic activity of NK cells against Huh7-HCVreplicon cells could not explain the findings in the blood of infected patients. We found a strong NK cell-mediated inhibition of HCV replication in the presence of minimal cytolysis as well as mild AST levels even at high E:T ratios, suggesting that cytotoxic activity of NK cells is not significantly involved in the defense against acute HCV infection at the very early stages. AST and ALT levels also did not differ when patients with acute HCV infection were analyzed separately with respect to the outcome of infection. Although we found CD56bright NK cells, the dominant intrahepatic NK cell subpopulation, to exert greater cytolytic activity in patients with acute resolving hepatitis C than in patients who developed chronic infection, we cannot draw conclusions on a role of these cells in the liver as we analyzed peripheral blood samples. Reports about “CD56Bright-killers” were made by Stegmann et. al. with TRAIL being the main cytotoxic molecule on these cells [54]. To measure the cytotoxic potency of the NK cells, we chose the Huh7-HCVreplicon cell system which, might be the closest model to study immune responses against a hepatotropic virus such as hepatitis C. In contrast, all previous studies on acute HCV mono-infection were conducted using hematopoietic cell lines such as K562 and/or 221 cells as targets for cytotoxicity.

The function of NK cells is critical dependent on the balance between inhibiting and activating NKRs. In recent studies of our group as well as that of Golden-Mason et al. it has been demonstrated that the activating NK cell receptor NKp46 is involved in antiviral activity of NK cells in chronic hepatitis C infection, since intrahepatic frequency

of this receptor was negatively correlated with hepatitis C viral loads [34], [61]. In our present experiments in acute HCV infection we found the prevalence of CD56bright NKp46+ NK cells to be significantly higher in resolvers than in the patients who progressed to chronical infection. Moreover, we found a positive correlation between the frequency of NKp46+ NK cells and inhibition of HCV replication as well as IFN-γ secretion. Finally, blocking of NKp46 with a specific antibody significantly decreased the ability of NK cells to inhibit viral replication *in vitro*, suggesting a role of this receptor in the early phase of acute HCV infection.

A further important component in anti-viral activity of NK cells, which has been analyzed in many previous studies on hepatitis C infection is NKG2D. We analyzed expression and function of NKG2D in NK cells in acute HCV infection, as it has been shown that HCV infection leads to induction of MHC-I-like ligands of NKG2D on infected hepatocytes [62]. Analogous to expression of NKp46, we found significantly higher surface expression of NKG2D in patients who spontaneously cleared the HCV infection as compared to those who developed a chronic course of infection. Moreover, we observed a strong correlation between expression of NKG2D and both NK cell-mediated inhibition of HCV replication and IFN-γ secretion. Finally, blocking NKG2D with a specific antibody significantly reduced the ability of NK cells to inhibit HCV replication *in vitro* strengthening a pivotal role of this receptor for resolution of acute HCV infection. Thus, our results provide circumstantial evidence that both activating receptors NKp46 and NKG2D are critically involved in NK cell functions, especially modulating IFN-γ production, in acute HCV/HIV co-infection. Consequently, spontaneous higher expression of both receptors may describe patients who are predisposed to naturally clear HCV infection in an IFN-γ dependent manner during the course of acute infection.

Previous studies analyzed the role of NK cells in acute HCV mono-infection and had controversial results. For example, Alter et al. found a correlation between low expression of NKp46 and NKG2D and spontaneous clearance of HCV infection, suggesting that this might be a consequence of receptor downregulation following NK cell activation by this receptor (NKG2D). Amadei et al. failed to demonstrate any correlation [29], [35]. Interestingly, Sene et al. described down regulation of NKG2D in chronic HCV mono-infection to lead to impaired IFN-γ secretion and NK cell cytotoxic activity as a potential viral escape mechanism of HCV.

Again these findings emphasize a pivotal role of this receptor for control of HCV infection by NK cells, which is in line with our results also in acute HCV infection [62]. Nevertheless, further analysis is required to understand the exact mechanisms, which result in up or down regulation of activating NK cell receptors in acute HCV infection. A critical issue in the interpretation of our results is the fact that HIV infection existed prior to acute hepatitis C. Thus, it remains unclear whether our results can be transferred to HCV mono-infection or whether they are specific for HIV/HCV coinfection. However, we could not observe any significant differences among our patients' characteristics, such as CD4+ T cell counts, age, gender, IL28B genotype and other parameters, which could explain the differences in NK cell phenotypes and functions on the outcome of acute HCV infection. HIV mono-infection alone has been described to lead to alterations in NK cell phenotype and function. Although all HIV positive patients in our studies were under efficient antiretroviral therapy, changes in NK cell biology due to HIV infection were not fully reversed; so that at least some of our observations are likely to be specific for HIV/HCV co-infection.

CD3(+) CD56(+) NK-like T cells are lymphocytes, which share characteristics from both NK cells and T cells [39], [40]. Of note, also this cell type has been described to control the outcome of acute HCV mono infection [41]. The reports on NK-like T cells and our present findings in NK cells, encouraged us to analyze the CD3(+) CD56(+) NK-like T cells also in HIV patients with acute hepatitis C. In our experiments, we wanted to clarify whether NK-like T cells also contribute to the mechanisms that affect outcomes of acute HCV infection in HIV+ patients.

IFN-γ is critically involved in the anti-HCV activity of lymphocyte populations [34], [57]. Here our work, demonstrates that IFN-γ is also a key element how CD3(+) CD56(+) NK-like T cells contribute to anti-HCV activity in early HCV infection. First, we showed that IL-12/IL-15 stimulated NK-like T cells from healthy individuals can effectively block HCV replication after co-incubation with Huh7-HCVreplicon cells. Secondly, we found that supernatants from NK-like T cells also efficiently inhibit HCV replication indicating that anti-HCV activity of these cells is not mediated by a cell-cell contact dependent mechanism. This observation was further confirmed, because we found minimal cytolytic activity as well as low AST levels after co-incubation of NK-like T cells with Huh7-HCVreplicon cells even at high E:T ratios.

Third, we observed that stimulated NK-like T cells produced significantly more IFN-γ than unstimulated cells and blocking of IFN-γ with a specific antibody significantly decreased the ability of NK-like T cells to inhibit HCV replication *in vitro*. Despite blocking of IFN-γ a substantial anti-HCV activity of NK-like T cells was found suggesting that other soluble factors than IFN-γ to be involved in antiviral activity.

HIV infection has already been described to be associated with phenotypical and functional changes of CD3(+) CD56(+) NK-like T cells [45], [46]. Furthermore, Montoya et al. showed a shift in NK-like T cell pool, because they found significantly decreased numbers of NK-like T cells in HIV patients compared with healthy individuals [46]. Indeed, we could not observe any similar alterations in our studied groups with respect to the frequency of NK-like T cells. Moreover, we observed functional dysregulation of NK-like T cells to be correlated with the HIV infection, because we found NK-like T cells from HIV patients to display significantly decreased IFN-γ production compared with healthy individuals. Our observation is in line with a prior report presented by Jiang et al. [45] who suggested that HIV infection was linked to chronic immune activation, which further leads to functional inactivation. On the other hand, in acute HCV monoinfection Golden Mason et al. showed a correlation between an activated phenotype of CD3(+) CD56(+) NK-like T cells and outcome of acute hepatitis C, which was favoring spontaneous clearance of infection [41]. Corresponding to this observation, we found CD69 to be significantly higher expressed on NK-like T cells from patients with a self-limited course of acute HCV infection than in HIV+ patients who subsequently developed chronic course. Since we could not find any correlation between CD69 expression and NK-like T cell functions and intrahepatic cells were not available to check the activity of NK-like T cells in the liver, it remains unclear whether NK-like T cells display an activated phenotype in acute hepatitis C. Thus, higher expression of CD69 in the periphery could just represent unspecific immune activation. More importantly, our findings do not support major participation of NK-like T cells in spontaneous clearance of acute hepatitis C in HIV patients, because *in vitro* we found IFN-γ production to play a significant role for anti-HCV activity, while at the same time NK-like T cells from HIV(+) patients displayed impaired IFN-γ production.

Taken together our results indicate that during the acute phase of hepatitis C infection in HIV positive patients a strong IFN-γ response mediates inhibition of HCV replication. Release of IFN-γ by NK cells is correlated with spontaneous clearance of HCV infection. This IFN-γ secretion from NK cells is critically regulated via the activating receptors NKp46 and NKG2D. Expression of these receptors defines NK cell subpopulations with strong anti-HCV capacity. Although NK-like T cells can also inhibit HCV replication *in vitro*, HIV infection seems to dysregulate the *in vivo* capacity of NK-like T cells, so that this cell population can contribute only little to anti-HCV defense in HIV infection.

6 References

[1] U. K. Widmer, A. Villaverde, and P. J. Grob, “[Epidemiology of hepatitis 1977-1979],” *Schweiz Med Wochenschr*, vol. 110, no. 24, pp. 930–937, Jun. 1980.

[2] Q. L. Choo, G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton, “Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome,” *Science*, vol. 244, no. 4902, pp. 359–362, Apr. 1989.

[3] Murphy DG, Sablon E, Chamberland J, Fournier E, Dandavino R, Tremblay CL. 2015. Hepatitis C virus genotype 7, a new genotype originating from Central Africa. J Clin Microbiol 53:967–972. doi:10.1128/JCM.02831-14.

[4] “WHO | Hepatitis C,” *WHO*. [Online]. Available: http://www.who.int/mediacentre/factsheets/fs164/en/.

[5] H. J. Alter *et al.*, “Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis,” *N. Engl. J. Med.*, vol. 321, no. 22, pp. 1494–1500, Nov. 1989.

[6] G. M. Robinson, J. N. Reynolds, and B. J. Robinson, “Hepatitis C prevalence and needle/syringe sharing behaviours in recent onset injecting drug users,” *N. Z. Med. J.*, vol. 108, no. 996, pp. 103–105, Mar. 1995.

[7] R. A. Tohme and S. D. Holmberg, “Is sexual contact a major mode of hepatitis C virus transmission?,” *Hepatology*, vol. 52, no. 4, pp. 1497–1505, Oct. 2010.

[8] F. Barré-Sinoussi *et al.*, “Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). 1983,” *Rev. Invest. Clin.*, vol. 56, no. 2, pp. 126–129, Apr. 2004.

[9] Kirchhoff F. „Optimale“ Anpassung pandemischer HIV-1-Stämme an den Menschen. BIOspektrum | 02.10 | 16. Jahrgang http://biospektrum.de/blatt/d_bs_pdf&_id=1024689.

[10] “WHO | HIV/AIDS,” WHO. [Online]. Available: http://www.who.int/mediacentre/factsheets/fs360/en/.

[11] Hoffmann C, Rockstroh J.K. HIV 2011. www.hivbuch.de S.4, S.7, S.536 http://hivbuch.files.wordpress.com/2011/12/hiv-20111.pdf

[12] C. S. Graham *et al.*, “Influence of human immunodeficiency virus infection on the course of hepatitis C virus infection: a meta-analysis,” *Clin. Infect. Dis.*, vol. 33, no. 4, pp. 562–569, Aug. 2001.

[13] Y. Benhamou *et al.*, “Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. The Multivirc Group,” *Hepatology*, vol. 30, no. 4, pp. 1054–1058, Oct. 1999.

[14] I. Bica *et al.*, “Increasing mortality due to end-stage liver disease in patients with human immunodeficiency virus infection,” *Clin. Infect. Dis.*, vol. 32, no. 3, pp. 492–497, Feb. 2001.

[15] M. A. Cooper, T. A. Fehniger, and M. A. Caligiuri, “The biology of human natural killer-cell subsets,” *Trends Immunol.*, vol. 22, no. 11, pp. 633–640, Nov. 2001.

[16] K. Hata, D. H. Van Thiel, R. B. Herberman, and T. L. Whiteside, “Natural killer activity of human liver-derived lymphocytes in various liver diseases,” *Hepatology*, vol. 14, no. 3, pp. 495–503, Sep. 1991.

[17] G. Trinchieri, “Biology of natural killer cells,” *Adv. Immunol.*, vol. 47, pp. 187–376, 1989.

[18] T. L. Whiteside and R. B. Herberman, “Role of human natural killer cells in health and disease,” *Clin. Diagn. Lab. Immunol.*, vol. 1, no. 2, pp. 125–133, Mar. 1994.

[19] A. Moretta *et al.*, “Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis,” *Annu. Rev. Immunol.*, vol. 19, pp. 197–223, 2001.

[20] A. Moretta, R. Biassoni, C. Bottino, M. C. Mingari, and L. Moretta, “Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis,” *Immunol. Today*, vol. 21, no. 5, pp. 228–234, May 2000.

[21] R. Gazit *et al.*, “Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1,” *Nat. Immunol.*, vol. 7, no. 5, pp. 517–523, May 2006.

[22] O. Mandelboim *et al.*, “Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells,” *Nature*, vol. 409, no. 6823, pp. 1055–1060, Feb. 2001.

[23] F. Borrego, M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks, “Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis,” *J. Exp. Med.*, vol. 187, no. 5, pp. 813–818, Mar. 1998.

[24] V. M. Braud *et al.*, “HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C,” *Nature*, vol. 391, no. 6669, pp. 795–799, Feb. 1998.

[25] N. Lee *et al.*, “HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A,” *Proc. Natl. Acad. Sci. U.S.A.*, vol. 95, no. 9, pp. 5199–5204, Apr. 1998.

[26] S. Bauer *et al.*, “Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA,” *Science*, vol. 285, no. 5428, pp. 727–729, Jul. 1999.

[27] M. Kubin *et al.*, “ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells,” *Eur. J. Immunol.*, vol. 31, no. 5, pp. 1428–1437, May 2001.

[28] D. Cosman *et al.*, “ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor,” *Immunity*, vol. 14, no. 2, pp. 123–133, Feb. 2001.

[29] B. Amadei *et al.*, “Activation of natural killer cells during acute infection with hepatitis C virus,” *Gastroenterology*, vol. 138, no. 4, pp. 1536–1545, Apr. 2010.

[30] S. I. Khakoo *et al.*, “HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection,” *Science*, vol. 305, no. 5685, pp. 872–874, Aug. 2004.

[31] A. Houldsworth *et al.*, “Polymorphisms in the IL-12B gene and outcome of HCV infection,” *J. Interferon Cytokine Res.*, vol. 25, no. 5, pp. 271–276, May 2005.

[32] P. An, C. L. Thio, G. D. Kirk, S. Donfield, J. J. Goedert, and C. A. Winkler, “Regulatory polymorphisms in the interleukin-18 promoter are associated with hepatitis C virus clearance,” *J. Infect. Dis.*, vol. 198, no. 8, pp. 1159–1165, Oct. 2008.

[33] J. Larkin, A. Bost, J. I. Glass, and S.-L. Tan, “Cytokine-activated natural killer cells exert direct killing of hepatoma cells harboring hepatitis C virus replicons,” *J. Interferon Cytokine Res.*, vol. 26, no. 12, pp. 854–865, Dec. 2006.

[34] B. Krämer *et al.*, “Natural killer p46High expression defines a natural killer cell subset that is potentially involved in control of hepatitis C virus replication and modulation of liver fibrosis,” *Hepatology*, vol. 56, no. 4, pp. 1201–1213, Oct. 2012.

[35] G. Alter *et al.*, “Reduced frequencies of NKp30+NKp46+, CD161+, and NKG2D+ NK cells in acute HCV infection may predict viral clearance,” *J. Hepatol.*, vol. 55, no. 2, pp. 278–288, Aug. 2011.

[36] S. Pelletier, C. Drouin, N. Bédard, S. I. Khakoo, J. Bruneau, and N. H. Shoukry, “Increased degranulation of natural killer cells during acute HCV correlates with the magnitude of virus-specific T cell responses,” *J. Hepatol.*, vol. 53, no. 5, pp. 805–816, Nov. 2010.

[37] M. Kronenberg, “Toward an understanding of NKT cell biology: progress and paradoxes,” *Annu. Rev. Immunol.*, vol. 23, pp. 877–900, 2005.

[38] D. G. Doherty and C. O’Farrelly, “Innate and adaptive lymphoid cells in the human liver,” *Immunol. Rev.*, vol. 174, pp. 5–20, Apr. 2000.

[39] P. H. Lu and R. S. Negrin, “A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency,” *J. Immunol.*, vol. 153, no. 4, pp. 1687–1696, Aug. 1994.

[40] D. I. Godfrey, H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer, “NKT cells: what’s in a name?,” *Nat. Rev. Immunol.*, vol. 4, no. 3, pp. 231–237, 2004.

[41] L. Golden-Mason, N. Castelblanco, C. O’Farrelly, and H. R. Rosen, “Phenotypic and functional changes of cytotoxic CD56pos natural T cells determine outcome of acute hepatitis C virus infection,” *J. Virol.*, vol. 81, no. 17, pp. 9292–9298, Sep. 2007.

[42] N. Kawarabayashi *et al.*, “Decrease of CD56(+)T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma,” *Hepatology*, vol. 32, no. 5, pp. 962–969, Nov. 2000.

[43] J. M. Werner *et al.*, “Innate immune responses in hepatitis C virus-exposed healthcare workers who do not develop acute infection,” *Hepatology*, vol. 58, no. 5, pp. 1621–1631, Nov. 2013.

[44] E. Jouvin-Marche *et al.*, “Lymphocytes degranulation in liver in hepatitis C virus carriers is associated with IFNL4 polymorphisms and ALT levels,” *J. Infect. Dis.*, vol. 209, no. 12, pp. 1907–1915, Jun. 2014.

[45] Y. Jiang *et al.*, “The function of CD3+CD56+ NKT-like cells in HIV-infected individuals,” *Biomed Res Int*, vol. 2014, p. 863625, 2014.

[46] C. J. Montoya, P. A. Velilla, C. Chougnet, A. L. Landay, and M. T. Rugeles, “Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals,” *Clin. Immunol.*, vol. 120, no. 2, pp. 138–146, Aug. 2006.

[47] A. Böyum, “Separation of leukocytes from blood and bone marrow. Introduction,” *Scand. J. Clin. Lab. Invest. Suppl.*, vol. 97, p. 7, 1968.

[48] G. Alter, J. M. Malenfant, and M. Altfeld, “CD107a as a functional marker for the identification of natural killer cell activity,” *J. Immunol. Methods*, vol. 294, no. 1–2, pp. 15–22, Nov. 2004.

[49] V. Lohmann, F. Körner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager, “Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line,” *Science*, vol. 285, no. 5424, pp. 110–113, Jul. 1999.

[50] N. Krieger, V. Lohmann, and R. Bartenschlager, “Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations,” *J. Virol.*, vol. 75, no. 10, pp. 4614–4624, May 2001.

[51] M. M. Dring *et al.*, “Innate immune genes synergize to predict increased risk of chronic disease in hepatitis C virus infection,” *Proc. Natl. Acad. Sci. U.S.A.*, vol. 108, no. 14, pp. 5736–5741, Apr. 2011.

[52] S. Knapp *et al.*, “Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus,” *Hepatology*, vol. 51, no. 4, pp. 1168–1175, Apr. 2010.

[53] A. Rauch *et al.*, “Influence of inhibitory killer immunoglobulin-like receptors and their HLA-C ligands on resolving hepatitis C virus infection,” *Tissue Antigens*, vol. 69 Suppl 1, pp. 237–240, Apr. 2007.

[54] K. A. Stegmann *et al.*, “Interferon-alpha-induced TRAIL on natural killer cells is associated with control of hepatitis C virus infection,” *Gastroenterology*, vol. 138, no. 5, pp. 1885–1897, May 2010.

[55] G. Ahlenstiel *et al.*, “Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C,” *Gastroenterology*, vol. 141, no. 4, pp. 1231–1239, 1239.e1–2, Oct. 2011.

[56] C. Romagnani *et al.*, “CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation,” *J. Immunol.*, vol. 178, no. 8, pp. 4947–4955, Apr. 2007.

[57] J. Jo *et al.*, “Analysis of CD8+ T-cell-mediated inhibition of hepatitis C virus replication using a novel immunological model,” *Gastroenterology*, vol. 136, no. 4, pp. 1391–1401, Apr. 2009.

[58] R. Thimme, D. Oldach, K. M. Chang, C. Steiger, S. C. Ray, and F. V. Chisari, “Determinants of viral clearance and persistence during acute hepatitis C virus infection,” *J. Exp. Med.*, vol. 194, no. 10, pp. 1395–1406, Nov. 2001.

[59] N. H. Shoukry *et al.*, “Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection,” *J. Exp. Med.*, vol. 197, no. 12, pp. 1645–1655, Jun. 2003.

[60] D. G. Bowen and C. M. Walker, “Adaptive immune responses in acute and chronic hepatitis C virus infection,” *Nature*, vol. 436, no. 7053, pp. 946–952, Aug. 2005.

[61] L. Golden-Mason, A. E. L. Stone, K. M. Bambha, L. Cheng, and H. R. Rosen, “Race- and gender-related variation in natural killer p46 expression associated with differential anti-hepatitis C virus immunity,” *Hepatology*, vol. 56, no. 4, pp. 1214–1222, Oct. 2012.

[62] D. Sène *et al.*, “Hepatitis C virus (HCV) evades NKG2D-dependent NK cell responses through NS5A-mediated imbalance of inflammatory cytokines,” *PLoS Pathog.*, vol. 6, no. 11, p. e1001184, Nov. 2010.

7 Abbreviations

ADCC	antibody dependent cell-mediated cytotoxicity
AHC	acute hepatitis C
aHCV	acute hepatitis C virus
AIDS	acquired immune deficiency syndrome
ALT	Alanine transaminase
APC	Allophycocyanin
AST	Aspartate transaminase
BFA	Brefeldin A
BSA	bovine serum albumine
CD	cluster of differentiation
CHC	chronic hepatitis C
cHCV	chronic hepatitis C virus
cm	centimetre
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylenediaminetetraacetic acid
E:T	Effector:target
et al.	lat. et aliter
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	forward scatter
g	gravity (9,81 m/s2), gram
h	hour
HAART	highly active antiretroviral therapy
HCC	hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	Interferon
IL	Interleukin
IU	international units
K	Kilo

KIR	killer cell immunglobulin like receptors
l	Litre
LAMP-1	Lysosomal-associated membrane protein 1
M	Molar
m	Milli, Metre
µ	Micro
MACS	magnetic activated cell sorter
MHC	major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
Min	Minute
MSM	men who have sex with men
n	nano, number
NCR	natural cytotoxicity receptors
NEA	non-essential amino acids
NK-cells	Natural killer cells
NKT-cells	Natural killer T cells
n.s.	not significant
p	probability value
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	Phycoerythrin
PerCP	peridinin-chlorophyll-protein complex
RBV	Ribavirin
RFI	relative fluorescence intensity
Rh	recombinant human
RT	Room temperature
S	Second
SSC	side scatter
TGF	transforming growth factor
TNF	Tumor necrosis factor
ULBP	UL16 binding protein 1
WHO	World Health Organization
°C	degree Celsius

8 Publications

Articles

First author

CD3(+)CD56(+) Natural Killer-Like T Cells Display Anti-HCV Activity but Are Functionally Impaired in HIV(+) Patients With Acute Hepatitis C. J Acquir Immune Defic Syndr. 2015 Dec 1;70(4):338-46. doi: 10.1097/QAI.0000000000000793.
Kokordelis P, Krämer B, Boesecke C, Voigt E, Ingiliz P, Glässner A, Wolter F, Srassburg CP, Spengler U, Rockstroh JK, Nattermann J

An effective interferon-gamma-mediated inhibition of hepatitis C virus replication by natural killer cells is associated with spontaneous clearance of acute hepatitis C in human immunodeficiency virus-positive patients.
Hepatology. 2014 Mar;59(3):814-27. doi: 10.1002/hep.26782. Epub 2014 Jan 21.
Kokordelis P, Krämer B, Körner C, Boesecke C, Voigt E, Ingiliz P, Glässner A, Eisenhardt M, Wolter F, Kaczmarek D, Nischalke HD, Rockstroh JK, Spengler U, Nattermann J

Co-author

Impaired CD4$^+$ T cell stimulation of NK cell anti-fibrotic activity may contribute to accelerated liver fibrosis progression in HIV/HCV patients.
J Hepatol. 2013 Sep;59(3):427-33.doi: 10.1016/j.jhep.2013.04.029. Epub 2013 May 9.
Glässner A, Eisenhardt M, Kokordelis P, Krämer B, Wolter F, Nischalke HD, Boesecke C, Sauerbruch T, Rockstroh JK, Spengler U, Nattermann J.

CD27(+)CD56Bright natural killer cells may be involved in spontaneous clearance of acute hepatitis C in HIV-positive patients.
AIDS. 2014 Aug 24;28(13):1879-84. doi: 10.1097/QAD.0000000000000355.
Eisenhardt M, Glässner A, Wolter F, Krämer B, Kokordelis P, Nischalke HD, Boesecke C, Rockstroh JK, Spengler U, Nattermann J.

HIV mono-infection is associated with an impaired anti-hepatitis C virus activity of natural killer cells.
AIDS. 2016 Jan 28;30(3):355-63. doi: 10.1097/QAD.0000000000000963. Goeser F, Glässner A, Kokordelis P, Wolter F, Lutz P, Kaczmarek DJ, Schwarze-Zander C, Boesecke C, Strassburg CP, Rockstroh JK, Spengler U, Krämer B, Nattermann J.

Hepatitis C virus (HCV) RNA profiles among chronic HIV/HCV-coinfected individuals in ESPRIT; spontaneous HCV RNA clearance observed in nine individuals. HIV Med. 2017 Jul;18(6):430-434. doi: 10.1111/hiv.12466. Epub 2016 Oct 26. Grint D, Tedaldi E, Peters L, Mocroft A, Edlin B, Gallien S, Klinker H, Boesecke C, Kokordelis P, Rockstroh JK.

Alterations of the NK cell pool in HIV/HCV co-infection.
PLoS One. 2017 Apr 5;12(4):e0174465. doi: 10.1371/journal.pone.0174465. eCollection 2017.
Kaczmarek DJ, Kokordelis P, Krämer B, Glässner A, Wolter F, Goeser F, Lutz P, Schwarze-Zander C, Boesecke C, Strassburg CP, Rockstroh JK, Spengler U, Nattermann J.

Congress contributions

Oral presentations

DGVS 2012 Hamburg
Einfluss Natürlicher Killerzellen auf den Spontanverlauf einer akuten Hepatitis C Virusinfektion bei HIV(+) Patienten
Pavlos Kokordelis, Benjamin Krämer, Christoph Boesecke, Andreas Glässner, Marianne Eisenhardt, Franziska Wolter, Esther Voigt, Patrick Ingiliz, Tilman Sauerbruch, Jürgen Rockstroh, Ulrich Spengler und Jacob Nattermann

CROI 2014 Boston

CD3+CD56+ Natural Killer-like T cells in HIV+ patients with acute hepatitis C

Pavlos Kokordelis, Benjamin Krämer, Christoph Boesecke, Esther Voigt, Patrick Ingiliz, Andreas Glässner, Ulrich Spengler, Jürgen K. Rockstroh and Jacob Nattermann

Abstracts/Posters

AASLD 2012 Boston

Activation of Natural Killer Cells During Acute Infection With Hepatitis C Virus in HIV(+) Patients

Pavlos Kokordelis, Christian Körner, Benjamin Krämer, Christoph Boesecke, Esther Voigt, Andreas Glässner, Marianne Eisenhardt, Franziska Wolter, Hans Dieter Nischalke, Tilman Sauerbruch, Jürgen Rockstroh, Ulrich Spengler, and Jacob Nattermann

GASL 2013 Hannover

Eine effektive anti-virale NK Zell-Antwort ist bei HIV(+) Patienten mit einem selbst-limitierenden Verlauf einer akuten Hepatitis C assoziiert

Kokordelis P., Krämer B., Boesecke C., Glässner A., Eisenhardt M., Voigt E., Ingiliz P., Wolter F., Strassburg C., Rockstroh J. K., Spengler U., Nattermann J.

CROI 2013 Atlanta

Effective inhibition of HCV replication by NK cells is associated with spontaneous clearance of acute hepatitis C in HIV(+) patients

Pavlos Kokordelis, Benjamin Krämer, Christoph Boesecke, Christian Körner, Esther Voigt, Patrick Ingiliz, Marianne Eisenhardt, Andreas Glässner, Franziska Wolter, Ulrich Spengler, Jürgen K. Rockstroh, and Jacob Nattermann

EASL 2013 Amsterdam

NK cell secreted IFN–γ blocks HCV replication and may promote spontaneous clearance of acute hepatitis C in HIV(+) patients

Pavlos Kokordelis, Benjamin Krämer, Christoph Boesecke, Christian Körner, Esther Voigt, Patrick Ingiliz, Marianne Eisenhardt, Andreas Glässner, Franziska Wolter, Christian P. Strassburg, Ulrich Spengler, Jürgen K. Rockstroh and Jacob Nattermann

ImmunoSensation 2013 Bonn

An effective IFN-γ mediated inhibition of HCV replication by NK cells is associated with spontaneous clearance of acute hepatitis C in HIV(+) patients

Pavlos Kokordelis, Benjamin Krämer, Christian Körner, Christoph Boesecke, Esther Voigt, Patrick Ingiliz, Andreas Glässner, Marianne Eisenhardt, Franziska Wolter, Dominik Kaczmarek, Hans Dieter Nischalke, Jürgen K. Rockstroh, Ulrich Spengler and Jacob Nattermann

GASL 2014 Tübingen

Phänotypische und funktionelle Analyse von natürlichen Killer T (NKT)-Zellen bei HIV-Patienten mit einer akuten Hepatitis C

Kokordelis P., Krämer B., Boesecke C., Glässner A., Eisenhardt M., Voigt E., Ingiliz P., Wolter F., Strassburg C. P., Rockstroh J. K., Spengler U., Nattermann J.

CHEP 2014 Berlin

CD3+CD56+ Natural Killer-like T cells in HIV+ patients with acute hepatitis C

Pavlos Kokordelis, Benjamin Krämer, Christoph Boesecke, Esther Voigt, Patrick Ingiliz, Andreas Glässner, Ulrich Spengler, Jürgen K. Rockstroh and Jacob Nattermann

CROI 2015 Seattle

HIV Infection is Associated with an Impaired anti-HCV Activity of NK-like T Cells

Pavlos Kokordelis, Benjamin Krämer, Christoph Boesecke, Esther Voigt, Patrick Ingiliz, Andreas Glässner, Franziska Wolter, Ulrich Spengler, Jürgen K. Rockstroh and Jacob Nattermann

EASL 2015 Wien

CD3(+)CD56(+) NK-like T cells show reduces anti-viral activitiy in acutely HCV/HIV infected patients

Pavlos Kokordelis, Benjamin Krämer, Christoph Boesecke, Esther Voigt, Patrick Ingiliz, Andreas Glässner, Franziska Wolter, Christian P. Strassburg, Ulrich Spengler, Jürgen K. Rockstroh and Jacob Nattermann

DZIF 2015 Bonn

CD3(+)CD56(+) NK-like T Zellen zeigen anti-HCV Aktivität, sind aber in ihrer Funktion bei akuter HCV/HIV Infektion gestört.

Pavlos Kokordelis, Benjamin Krämer, Christoph Boesecke, Andreas Glässner, Franziska Wolter, Dominik Kaczmarek, Esther Voigt, Patrick Ingiliz, Christian P. Strassburg, Jürgen K. Rockstroh, Ulrich Spengler, und Jacob Nattermann

GASL 2015 München

Die HIV Infektion ist mit einer verminderten anti-HCV Aktivität von CD3+CD56+ NK-like T Zellen assoziiert

Kokordelis P., Krämer B., Boesecke C., Glässner A., Wolter F., Kaczmarek D, Voigt E., Ingiliz P., Strassburg C. P., Rockstroh J. K., Spengler U., Nattermann J.

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ζήτῳ ὡς ἀγαπάτε