Research Article

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Hypoxia impairs anti-viral activity of natural killer (NK) cells but has little effect on anti-fibrotic NK cell functions in hepatitis C virus infection

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Background & Aims: Natural killer (NK) cells have been shown to exert anti-viral as well as anti-fibrotic functions in hepatitis C virus (HCV) infection. Previous studies, however, analyzed NK cell functions exclusively under atmospheric oxygen conditions despite the fact that the liver microenvironment is hypoxic. Here, we analyzed the effects of low oxygen tension on anti-viral and anti-fibrotic NK cell activity.

Methods: Peripheral (n = 34) and intrahepatic (n = 15) NK cells from HCV(+) patients as well as circulating NK cells from healthy donors (n = 20) were studied with respect to anti-viral and anti-fibrotic activity via co-culture experiments with HuH7 replicon cells and hepatic stellate cells, respectively.

Results: Anti-viral activity of resting NK cells from healthy controls was not affected by hypoxia. However, hypoxia significantly reduced the response of healthy NK cells to cytokine stimulation. In contrast to healthy controls, we observed resting and cytokine activated peripheral NK cells from HCV patients to display a significantly decreased anti-viral activity when cultured at 5% or 1% oxygen, suggesting HCV NK cells to be very sensitive to hypoxia. These findings could be confirmed when intrahepatic NK cells were tested. Finally, we show that anti-fibrotic NK cell activity was not affected by low oxygen tension.

Conclusions: Our results show that anti-viral function of NK cells from HCV(+) patients is critically affected by a hypoxic microenvironment and, therefore, indicate that in order to obtain an accurate understanding of intrahepatic NK cell anti-HCV activity, the laboratory modelling should take into account the liver specific levels of oxygen.

Keywords: NK cells; Hepatic stellate cells; HuH7 replicon; NKp46; NKG2D; HCV; Intrahepatic; Liver-infiltrating lymphocytes; Hypoxia.

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Abbreviations: E:T ratio, effector:target ratio; FACS, fluorescence-activated cell sorting; HBV, hepatitis B virus; HCV, hepatitis C virus; HIF, hypoxia-inducible factor; HIV, human immunodeficiency virus; HSC, hepatic stellate cells; IFN, interferon; IL, interleukin; LIL, liver-infiltrating lymphocytes; NK cell, natural killer cell; NKR, NK cell receptor; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor; TPI1, triosephosphat isomerase 1; PDK1, pyruvate dehydrogenase kinase 1; PFKFBP3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis worldwide and remains a challenging health problem even after the emergence of highly effective and well-tolerated direct-acting antiviral drugs.

Immune responses critically modulate the natural course of both acute and chronic hepatitis C and increasing data indicate that natural killer (NK) cells play an especially important role in this context [1–4]. NK cells, a heterogeneous lymphocyte population, constitute a major component of the intrahepatic lymphocyte pool. A typical feature of NK cells is their ability to attack and kill malignant transformed and virus-infected cells without prior immunization. Moreover, NK cells are important producers of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ thereby mediating anti-viral and immune-modulating effects [5,6].

With respect to hepatitis C, several studies showed that NK cells are activated in the acute phase of infection [1,2,7] and we recently demonstrated that an effective antiviral NK cell response is associated with a self-limiting course of acute hepatitis C in HIV(+) patients [3]. Beyond their crucial role in antiviral immunity, NK cells also represent important regulators of hepatic fibrogenesis as they can kill activated hepatic stellate cells (HSC), thereby mediating anti-fibrotic effects [8–11]. Accordingly, we could show that a dysregulated NK cell activity is associated with advanced stages of HCV-associated liver fibrosis [9].

NK cell activity is tightly regulated by signals delivered by activating and inhibitory NK cell receptors (NKR) and their respective ligands [12]. With respect to NK cell-mediated inhibition of HCV replication, the activating NK cell receptors, NKG2D and NKp46 [3,13,14], are considered to play an important role. Moreover, NKp46 and NKG2D together with TRAIL and FasL have been shown to be involved in anti-fibrotic NK cell activity [9,13].

Table 1. Patient Characteristics.

	HCV(+) patients	Healthy controls
Number	38	20
Female sex[a)]	13 (33%)	12 (60%)
Age (years)[b)]	50.3 (21-77)	38.7 (27-75)
Clinical data		
ALT U/L[b)]	65.1 (9-212)	n.a.
AST U/L[b)]	54.9 (17-143)	n.a.
γ-GT [b)]	119.5 (24-566)	n.a.
HCV-status		
HCV load (x10^5 IU/ml)[b)]	12.8 (0-42)	n.a.
HCV-genotypes:		
Genotype 1[a)]	29 (76%)	-
Genotype 2[a)]	-	-
Genotype 3[a)]	5 (13%)	-
Genotype 4[a)]	2 (5%)	-
Undetermined genotype[a)]	2 (5%)	-

(a) number of cases (number/total in%). (b) Mean (range). n.a. – not analyzed.

However, all these data have been obtained in experiments performed under atmospheric oxygen concentrations (20% O_2), which may not reflect the *in vivo* situation.

The liver is the main site of viral replication in HCV infection and thus, the intrahepatic microenvironment is considered to importantly modulate immune responses in hepatitis C. Besides soluble molecules, such as hormones, growth factors, cytokines, or signals delivered from surrounding cells, oxygen concentrations in the tissue have been shown to modulate NK cell phenotype and functions [15,16].

Studies using polarographic techniques with oxygen electrodes indicate that under physiologic conditions the oxygen tension in the liver ranges from about 10 mmHg (corresponding to 1.3% O_2) near the central vein to 60 mmHg close to the portal vein (8% O_2), with a median tension of 40 mmHg (5% O_2) [17,18].

Moreover, oxygen levels may further decrease under inflammatory conditions [19,20] or due to vascular disorganization induced by hepatic injury and progressive fibrogenesis [21,22].

Although data obtained in tumor models indicate that NK cell function is significantly affected by hypoxia [15,16,23,24], no information is currently available regarding the potential impact of low oxygen concentrations on antiviral and antifibrotic NK cell activity.

Here, we show that hypoxia critically impairs antiviral NK cell functions, while direct cytotoxicity involved in antifibrotic NK cell activity remains unchanged. Furthermore, we show that both peripheral and intrahepatic NK cells from patients with chronic hepatitis C are significantly more susceptible to low oxygen tension than NK cells from healthy controls.

Patients and methods

Patients

A total of 38 HIV(−), HBV(−) patients with chronic hepatitis C were included. As a control, we analyzed 20 healthy blood donors. Detailed patient characteristics are given in Table 1.

Informed consent was obtained from all subjects. The study had been approved by the local ethics committee of the University of Bonn (Bonn, Germany).

Hypoxia

Hypoxic conditions (5% and 1% O_2) were obtained in a modular incubator chamber (purchased from Billups-Rothenberg Inc., Del Mar, CA) [25]. Both preincubation of NK cells and co-incubation with target cells was done in hypoxic conditions. See Supplementary methods for detailed description.

Flow cytometry

The dyes and fluorochrome-labeled antibodies that were used for fluorescence-activated cell sorting (FACS) analysis are described in the Supplementary methods. Samples were analyzed on a FACSCanto™ II flow cytometer using the CellQuest Pro (BD Biosciences) and FlowJo 7.2.2 (TreeStar Inc., Ashland, OR) software packages.

Liver specimens

Intrahepatic lymphocytes were isolated from explanted livers of HCV(+) patients (n = 15). For details see Supplementary methods.

NK cell separation and stimulation

NK cells were separated from peripheral blood mononuclear cells (PBMC) using the EasySep™ Human NK Cell Enrichment Kit (Stemcell Technologies, Grenoble, France) according to the manufacturer's protocol. The purity of NK cells was >95%.

Culture conditions and stimulation of NK cells are described in the Supplementary methods.

Hypoxyprobe assay

The Hypoxyprobe-1 Kit was purchased from Hypoxyprobe Inc. (Middlesex, MA). After incubation of NK cells in 20%, 5% or 1% oxygen (v/v) for 16 h, 200 μM pimonidazole (a hypoxia marker which forms protein adducts in a hypoxic cellular environment) was added to the cells for 2 h. Cells were harvested and pimonidazole adducts were detected via flow cytometry.

Cells

Huh7$_{A2}$HCV replicon cells were kindly provided by V. Lohmann and R. Bartenschlager (Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany). Isolated primary human HSC were obtained from ScienCell, (San Diego, CA, USA). Cells were cultured as described before [13]. Further details are given in Supplementary methods.

Functional analysis of NK cells

IFNγ production, degranulation and antiviral activity of NK cells as well as NK cell-induced HSC apoptosis were analyzed as described before [13]. Detailed experimental procedures are described in the Supplementary methods section.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 4.00; GraphPad Software Inc., La Jolla, CA). Statistical tests are stated in the figure legends. A two-sided p value <0.05 was considered significant.

qPCR of HIF1α, TPI1, PDK1, and PFKFBP3 genes

Total RNA was extracted from resting NK cell incubated at 20%, 5% or 1% O_2 using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

PCR was carried out on a LightCycler instrument using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA) and 0.5 μM of primers.