Research Article

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Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C*03:04 and modulate NK cell function

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Background & Aims: Both natural killer (NK) cells and human leukocyte antigen (HLA)/killer cell immunoglobulin like receptor (KIR) interactions have been shown to play an important role in the control, clearance and progression of hepatitis C virus (HCV) disease. Here we aimed at elucidating the effects of viral peptides derived from HCV on HLA stabilization, changes in KIR binding and primary NK cell function.

Methods: Transporter for antigen presentation-deficient 722.221 cells stably transfected with HLA-C*03:04 were used to screen 200 overlapping peptides, covering the non-structural protein 3 (NS3) and core protein of HCV genotype 1, for their ability to bind and stabilize HLA-C*03:04. Binding of KIR2DL3 to the HLA-peptide complex was assessed using a KIR2DL3-IgG fusion construct. Primary NK cells were isolated from healthy donors to investigate the effects of identified peptides on KIR2DL3$^+$ NK cell function.

Results: Thirty-one peptides able to stabilize HLA-C*03:04 were identified. One 9mer peptide, YIPLVGAPL, resulted in significantly higher KIR2DL3 binding to HLA-C*03:04$^+$ 722.221 cells and suppression of primary KIR2DL3$^+$ NK cell function. Interestingly this sequence exhibited a high frequency of mutations in different HCV genotypes. These genotype-specific peptides showed lower HLA-C*03:04 stabilization, decreased binding of the inhibitory KIR2DL3 and lower inhibition of NK cell function.

Conclusions: Taken together we show that a viral peptide derived from the core protein of HCV genotype 1 binding to HLA-C*03:04 results in a sequence-dependent engagement of the inhibitory NK cell receptor KIR2DL3, while the large majority of the remaining 30 HLA-C*03:04 binding HCV core peptides did not. These data show that sequence variations within HCV can modulate NK cell function, providing potential pathways for viral escape.

Lay summary: We identified a HCV peptide that dampens NK cell responses, and thereby possibly prevents killing of infected cells through this part of the innate immune system. This is facilitated via presentation of the viral peptide on HLA*03:04 to the inhibitory KIR receptor KIR2DL3 on NK cells. Naturally occurring sequence mutations in the peptide alter these interactions making the inhibition less efficient.

Introduction

Natural Killer (NK) cells play a pivotal role in the early defense against virus infections and malignancies [1,2], as they are part of the innate arm of the human immune system and able to respond rapidly against encountered pathogens without prior need of sensitization. The main NK cell function is the elimination of target cells by directed release of perforin and granzyme, or engagement of apoptosis-inducing receptors such as FAS [2]. Furthermore, NK cells can shape the following antigen-specific immune responses through the production of cytokines and chemokines or by interacting directly with other cells of the immune system, such as T cells, dendritic cells and monocytes [3,4].

Due to the potent nature of their effector functions, the activation of NK cells is tightly regulated via a plethora of activating and inhibiting receptors and their interaction with respective ligands [5,6]. One major group of receptors on NK cells are the killer cell immunoglobulin like receptors (KIRs), which interact mainly with human leukocyte antigen (HLA) class I molecules on the surfaces of other cells [7,8]. Both activating and inhibiting KIRs have been described. The binding of specific KIRs to their respective HLA ligand can be further modulated by the HLA class I-presented peptide [9]. These interactions have been shown to

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Abbreviations: HCV, hepatitis C virus; KIR, killer cell immunoglobulin like receptor; HLA, human leukocyte antigen; NK, natural killer; OLP, overlapping peptide.

play an important role in regulating the NK cell response against the human immunodeficiency virus (HIV), and also enable HIV-1 to escape NK cell recognition through the selection of viral sequence variations [10].

In the case of hepatitis virus infections, NK cells play an important role in the clearance of infection and disease progression [1]. A large number of studies have highlighted the importance of NK cells in viral hepatitis, focusing mainly on patients infected with hepatitis B virus (HBV) and hepatitis C virus (HCV) [11]. KIR-HLA interactions seem of particular importance in HCV infection as suggested by genetic association studies which linked HLA-C genotypes in conjunction with KIR2DL3 to viral clearance [12,13]. A more recent study further expanded on this, with the finding that KIR2DS3 is negatively associated with viral clearance [14]. The exact mechanism underlying these associations is not fully understood, but probably depends on the strength of the KIR/HLA interaction, which in case of KIR2DL3 and HLA-C alleles of the group 1 (HLA-C1) is rather weak, compared to other inhibition events [12]. HLA-C*03:04 is a common member of the HLA-C1 group, and is expressed at varying frequencies, ranging from 1% in Saudi Arabian populations all the way to 54% in Brazilians (allelfrequencies.net). In Caucasian populations in Germany, the range is between 14–21%. HLA-C*03:04 is a ligand for the above mentioned KIR2DL3, and the described role in HIV infection [16] might indicate further involvement in other viral infections.

While numerous HCV-derived $CD4^+$ and $CD8^+$ T cell epitopes have been described, no data are available on the role of HCV peptides presented by HLA class I molecules for KIR binding and KIR^+ NK cell function. Therefore, our aim was to investigate whether viral peptides derived from HCV core have an effect on the function of primary KIR^+ NK cells.

Materials and methods

Cell lines and human peripheral blood mononuclear cells

We used a previously described 721.221 human B-cell line, which has been stably transduced with ICP47, a herpes simplex virus (HSV) protein that blocks transporter for antigen presentation (TAP)-dependent loading of MHC class I molecules, and also transfected with HLA-C*03:04. Additionally we used the same cell line without ICP47 TAP-block. These cells were kept in RPMI medium 1640, supplemented with 10% heat-inactivated fetal calf serum, streptomycin and penicillin (R10). A *TAP*-knockout (KO) cell line based on the 721.221-C*03:04 cells was generated (221-C*03:04-*TAP1*-KO) using CRISPR/CAS technology [15] (plasmids were supplied by addgene). Cells were selected in R10 supplemented with puromycin, blasticidine and neomycin. Human primary blood mononuclear cells (PBMC) were isolated from healthy donors, using density centrifugation and either used directly or cryopreserved in liquid nitrogen until use. All donors gave informed consent.

HLA stabilization and KIR binding assay

HLA stabilization and KIR binding assays were performed as previously described [16]. In short, cells were washed with FBS free RPMI 1640 (R0) to remove any remaining foreign peptides from culturing in R10. Afterwards 721.221-ICP47-C*03:04, or 221-C*03:04-*TAP1*-KO cells were pulsed with 100 μM of the respective HCV peptide for 20 h at 26 °C. Previously described peptides that stabilized HLA-C*03:04 expression GAVDPLLAL (GAL) and GAVDPLLKL (GKL) [17], were used as positive controls, whereas culturing in the absence of peptides was used as negative control. After peptide-pulsing, cells were stained using an anti-HLA-ABC antibody (clone W6/32), fixed in 4% paraformaldehyde and analyzed using flow cytometry. KIR binding was analyzed after 20 h of HLA stabilization, as described above, with the respective peptides of interest. Cells were stained using KIR2DL3-Fc chimera (R&D), for 1 h on ice, secondary staining with anti-human FC antibody was performed for 30 min on ice. Afterwards cells were fixed and analyzed using flow cytometry.

NK cell degranulation assay

Primary NK cells were isolated from PBMCs of healthy donors, using Ficoll-Hypaque centrifugation, and rested overnight in R10 supplemented with 1 ng/ml IL-15. NK cells were co-incubated with peptide-pulsed 721.221-ICP47-C*03:04 cells at an effector to target (E:T) ratio of 5:1 in the presence of 3 μl anti-CD107a in 96 well plates. After 1 h incubation at 37 °C, GolgiPlug™ (BD) was added followed by 5 additional hours of incubation at 26 °C. Cells were subsequently stained using anti-CD3, anti-CD16, anti-CD56 and anti-KIR2DL3 for 30 min at 4 °C, fixed using 4% paraformaldehyde in PBS for 30 min and analyzed by flow cytometry. The gating strategy to identify responses in $KIR2DL3^+$ and $KIR2DL3^-$ cells is shown in Supplementary Fig. 1.

Data acquisition, analysis and statistics

Flow cytometry was performed on an LSRFortessa™ and FACSCanto™ II (BD Bioscience) and analyzed using FlowJo software v10 (Tree Star, Inc.). Figures were designed and statistical analysis done using GraphPad Prism 5 (GraphPad Software, Inc.). All values in bar graphs represent mean ± SEM unless stated otherwise. Association of KIR2DL3 with HCV genotype was performed in GraphPad using the latest metadata [18] and data on KIR distribution in different ethnicities, kindly provided by Mary Carrington.

Results

*Several peptides derived from the core protein and non-structural protein 3 of HCV bind to HLA-C*03:04*

The goal of this study was to investigate the effect of HCV core and non-structural protein 3 (NS3)-derived epitopes presented by HLA-C*03:04 on binding to KIR2DL3 and modulation of NK cell activation. To identify suitable epitopes that are presented by HLA-C*03:04, we screened a pool of 200 overlapping peptides (OLP) of 15 amino acid length and overlapping by 11 amino acids, spanning both core and NS3 of HCV genotype 1. We identified 31 peptides that stabilized HLA-C*03:04 (Fig. 1) on the surface of 721.221-ICP47-C*03:04 cells. We subsequently focused on those peptides that showed a clear increase in HLA-C*03:04 expression, defined by having a MFI higher than the mean value plus 2 standard deviations of all non-stabilizing peptides for further assessment, resulting in ten 15mer peptides (Fig. 1 and Supplementary Fig. 2).

As the optimal length of a peptide binding to HLA class I molecules is between 9–11 amino acids, we attempted to predict the optimal binding sequence for HLA-C*03:04 within these ten 15mer peptides, based on previously published data and binding motifs (Table 1). These 9mer peptides were compared to the original 15mer peptides for their ability to stabilize HLA-C*03:04 expression (Table 1).

Two of the newly synthesized 9mer peptides, 34* and 120*, showed a markedly higher stabilization of HLA-C*03:04 compared to the original 15mer, while the other 9mer peptides showed no improved or even reduced HLA stabilization capacity. These two 9mer peptides and the original ten 15mer peptides were subsequently used to investigate their impact on the binding of KIR2DL3 to the HLA-peptide complex. We performed titration experiments to identify the optimal concentration to be used in later assays (Supplementary Fig. 4). Taken together, we identified 10 novel HCV peptides that bound to HLA-C*03:04.