Research Article

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Increased degranulation of natural killer cells during acute HCV correlates with the magnitude of virus-specific T cell responses

Sandy Pelletier[1,3], Christian Drouin[1,3], Nathalie Bédard[1], Salim I. Khakoo[2], Julie Bruneau[1,4], Naglaa H. Shoukry[1,5,*]

[1]*Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Hôpital St-Luc, Montréal, QC, Canada;* [2]*Department of Hepatology, Division of Medicine, Imperial College, London, UK;* [3]*Département de microbiologie et immunologie, Université de Montréal, Montréal, QC, Canada;* [4]*Département de médecine familiale, Université de Montréal, Montréal, QC, Canada;* [5]*Département de médecine, Université de Montréal, Montréal, QC, Canada*

Background & Aims: Natural killer (NK) cells provide early defense against viral infections by killing infected cells and producing cytokines that inhibit viral replication. NK cells also interact with dendritic cells (DCs) and this reciprocal interaction regulates both innate and adaptive immunity. Genetic studies have suggested that NK cell activity is a determinant of HCV infectious outcome but a functional correlation has not been established. We hypothesized that increased NK cell activity during acute HCV infection correlates with spontaneous viral clearance.

Methods: We used multiparametric flow cytometry to monitor longitudinally the phenotype and the activity of NK cells in a cohort of intravenous drug users following HCV exposure. Three groups were studied: acute HCV with chronic evolution ($n = 13$), acute resolving HCV ($n = 11$), and exposed un-infected individuals ($n = 10$). We examined the expression of several NK cell-activating and -inhibiting receptors, IFN-γ production and CD107a degranulation upon stimulation, and the kinetics of NK cell responses relative to T cell responses.

Results: We observed decreased expression of the inhibitory NKG2A receptor in NK cells following spontaneous HCV clearance. In addition, we observed increased NK cell degranulation during acute HCV irrespective of infectious outcome. NK cell peak responses preceded or coincided with peak T cell responses. Furthermore, NK cell degranulation correlated with the magnitude of HCV-specific T cells.

Conclusions: Our results demonstrate that NK cells are activated during acute HCV regardless of infection outcome and may play an indirect role through induction and priming of T cell responses.

Keywords: Innate immunity; Viral hepatitis; Cytotoxicity; Cytokines.
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*Corresponding author. Address: Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Hôpital St-Luc, 264 Boul., René-Lévesque Est, Local PEA-316, Montréal, QC, Canada H2X 1P1.
E-mail address: naglaa.shoukry@umontreal.ca (N.H. Shoukry).
Abbreviations: DC, dendritic cells; ELISPOT, enzyme-linked immunospot assay; HBV, hepatitis B virus; HCV, hepatitis C Virus; HIV, human immunodeficiency virus; ICS, intracellular cytokine staining; IDUs, intravenous drug users; IFN-γ, interferon gamma; NK, natural killer cells; PBMC, peripheral blood mononuclear cells.

Introduction

The majority of individuals exposed to hepatitis C virus (HCV) develop persistent infection and chronic liver disease [1]. Acute HCV is characterized by a significant delay in the onset of adaptive T cell responses despite its active viral replication. This suggests a failure of innate immunity to contain viral replication and provide the necessary signals to prime an efficient adaptive immunity critical to spontaneous viral clearance [2,3]. Natural killer (NK) cells are the most important effector population of the innate immune response. Two NK cell subsets can be distinguished based on their differential expression of CD56 and CD16: immunoregulatory $CD3^{-}CD56^{bright}CD16^{-}$ and cytolytic $CD3^{-}CD56^{dim}CD16^{+}$ [4]. NK cells provide an early defense line against viral infections by killing infected cells and producing cytokines that can directly inhibit viral replication and trigger the adaptive immune response. NK cells use inhibiting and activating receptors as a mean of controlling their activity. NK cells interact with dendritic cells (DCs) and this reciprocal interaction results in the regulation of both innate and adaptive immune responses [5,6]. DCs can activate NK cells by binding to NKp30 on the surface of NK cells and by secreting numerous cytokines such as IL-12 [7]. NK cells, in turn, secrete IFN-γ and TNF-α which induce DC maturation and trigger the adaptive immune response [8]. In addition, NK cells can also kill immature DCs and inhibit their capacity to prime or tolerize adaptive T cell responses [5,9].

Two observations highlighted the potential role of NK cells during the early phase of HCV infection. First, HCV surface glycoprotein E2 can bind CD81 on the surface of NK cells and inhibit cytotoxicity and IFN-γ production [10,11]; however, Yoon et al. have recently demonstrated that the exposure of NK cells from healthy donors to *in vitro*-produced HCV virions did not influence their function [12]. Second, genes encoding the inhibitory NK cell receptor killer-cell immunoglobulin like receptor (KIR)2DL3 and its human leukocyte antigen C group 1 (HLA-C1) ligand, directly influence the resolution of HCV infection in individuals homozygous for these genes [13,14]. These observations suggest that the

Research Article

inhibition of NK function during the early phase of HCV may contribute to viral persistence.

Several groups have studied NK cells during chronic HCV infection but the results regarding NK cell frequency, cytotoxicity, cytokine production, and receptor expression are conflicting [15–20]. This probably reflects the complexity of activating and inhibiting signals that control NK cells. Only one study has compared NK cell function in chronic HCV patients with spontaneous resolvers from a single source outbreak [15]. The authors of the study demonstrated that the frequency of the $CD56^{dim}$ NK cell subset was decreased in individuals with chronic HCV, and that NK cells expressed the NKG2A/C/E receptors at higher frequency [15]; however, the activity of NK cells during acute HCV, when their role would be most prominent, and its correlation with the infectious outcome, were not studied.

In this study, we used multiparametric flow cytometry to monitor longitudinally the phenotypic and functional changes in NK cells from a unique cohort of intravenous drug users (IDUs) at high-risk of HCV infection before and during acute HCV infections that progressed to spontaneous resolution or viral persistence. In addition, we monitored NK cells activity in a group of HCV-exposed but un-infected individuals. We demonstrated that NK cell degranulation is increased during acute HCV, regardless of the infection outcome. We also observed a decline in NKG2A expression in NK cells following spontaneous viral clearance, and CD161 expression in infections progressing to chronicity. Finally, we showed that NK cell response peaks prior to T cell response and that NK cell degranulation correlate with the magnitude of the HCV-specific T cell response, suggesting an indirect role for NK cells in priming adaptive immune responses.

Patients and methods

Study subjects and clinical follow-up

A total of 34 HCV-exposed individuals and 10 normal donors were included in this study. HCV acutely infected subjects were recruited among high-risk IDUs participating in the Montreal Acute HepC cohort study (HEPCO) [21], the methadone treatment, and the Hepatology clinics at St-Luc hospital of the Centre Hospitalier de l'Université de Montréal (CHUM). This study was approved by the institutional ethics committee (Protocols # SL05.014 and SL05.025) and conducted according to the Declaration of Helsinki. All participants signed an informed consent upon enrolment. Acute HCV infection ($n = 24$) was defined as detection of positive HCV RNA and/or HCV antibodies following a previous negative test in the past 6 months, or a positive HCV RNA with concomitant negative HCV antibodies tests. The mean follow-up interval between the last aviremic and the first viremic time point was 12 weeks (range: 1–25 weeks), and the estimated time of the infection was defined as the median between the last aviremic and the first viremic time point. The duration of the infection was defined as weeks after the estimated time of infection. Spontaneous viral resolution ($n = 11$) or persistent infection ($n = 13$) was defined as the absence or presence of HCV RNA at 12 weeks post enrolment. This classification is based on recent Canadian guidelines that recommend IFN therapy to all HCV acutely infected patients if they remain HCV positive by week 12 [22]. All patients included in this study were either ineligible or refused IFN therapy. Their classification as acute or chronic did not change whether they were classified at week 12 or 24. Exposed uninfected ($n = 10$) are IDUs who have admitted sharing a needle or injection materials with an HCV-infected individual but remained HCV RNA and HCV antibody negative. In this study, three time points were analyzed for each patient representing three phases of HCV infection: pre-infection baseline, acute HCV, and follow-up. Baseline was defined as time before HCV infection or reported needle sharing for exposed un-infected (range: −1 to −22 weeks; mean −10 weeks). Baseline samples were available for eight chronic patients, five spontaneous resolvers, and five exposed un-infected. Acute HCV was defined as 12 weeks (range: 6–17 weeks) post estimated time of infection for HCV-infected individuals, and 4 weeks (range: 0–12 weeks) after needle sharing for exposed un-infected. The follow-up time point was defined as 52 weeks (range: 34–62 weeks) post- estimated time of infection for HCV-infected individuals or 36 weeks (range: 20–56 weeks) after needle sharing for exposed un-infected. All patients tested negative for human immunodeficiency virus (HIV) and hepatitis B virus (HBV).

HCV RNA testing and quantification

Qualitative HCV RNA tests were performed using an automated COBAS AmpliPrep/COBAS Amplicor HCV test, version 2.0 (sensitivity, 50 IU/ml) (Roche Molecular Systems, Inc., Branchburg, NJ). HCV genotyping was done using standard sequencing for the *NS5B* region, and was performed by the Laboratoire de Santé Publique du Québec (Ste-Anne-de-Bellevue, QC, Canada) as part of the clinical follow-up of patients. Additional HCV RNA quantification was performed using an in-house quantitative real-time reverse transcription-PCR assay, as previously described [23].

Flow cytometry antibodies and reagents

Directly conjugated antibodies against the following molecules were used: CD3-Pacific Blue (clone UCHT1), CD16-allophycocyanin (APC)-Cy7 (clone 3G8) or CD16-APC-H7 (clone 3G8), CD56-phycoerythryin (PE)-Cy7 (clone B159), CD107a-PE-Cy5 (clone H4A3), CD158a-fluorescein isothiocyanate (FITC) (clone HP-3E4), CD158b-FITC (clone CH-L), CD161-PE-Cy5 (clone DX12), NKB1-FITC (clone DX9), NKG2D-APC (clone 1D11), NKp30-Alexa 647 (clone P30-15), and IFN-γ-APC (clone B27) (all from BD Biosciences, San Jose, CA, USA); CD69-PE-Texas Red (ECD) (clone TP1-55-3), and NKp44-PE (clone Z231) (both from Beckman Coulter, Marseille, France); NKG2A-PE (clone #131411) (from R&D Systems, Minneapolis, MN, USA). Live cells were identified using an Aqua Live/Dead fixable dead cell Stain Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. We have used four phenotypic panels and one functional panel. "Fluorescence minus one" control stains were used to determine background levels of staining. Multiparameter flow cytometry was performed using a standard BD LSR II instrument equipped with blue (488 nm), red (633 nm), and violet (405 nm) lasers (BD Biosciences) to systematically perform seven-color staining using FACSDiva software (BD Biosciences). Compensation was performed with single fluorochromes and BD CompBeads (BD Biosciences). Data files were analyzed using FlowJo software, version 8.6.3 for Mac (Tree Star, Inc., Ashland, OR).

Multiparametric phenotypic characterization of NK cells

All flow cytometry assays were performed on cryopreserved samples. For phenotypic analysis, $1–2 \times 10^6$ peripheral blood mononuclear cells (PBMCs) were stained with surface antibodies for 30 min at 4 °C and washed twice in fluorescence-activated cell sorting (FACS) buffer (1× phosphate-buffered saline [PBS], 1% fetal bovine serum [FBS], 0.02% NaN_3), and fixed in FACS fix buffer (1× PBS, 1% formaldehyde).

Intracellular cytokine staining (ICS) and CD107a degranulation assay

PBMCs (2×10^6) were incubated with anti-CD107a antibody and either culture medium as a negative control, or K562 leukemia target cell line (ATCC, Manassas, VA, USA) at 37 °C in R-10 medium (RPMI medium [Invitrogen, Carlsbad, CA, USA] supplemented with 10% FBS). Following 1 h of stimulation, 10 μg/ml of brefeldin A (Sigma–Aldrich, St-Louis, MO, USA) and 6 μg/ml of monensin sodium salt (Sigma–Aldrich) were added, and cells were then incubated for a total of 6 h. Cells were washed with FACS buffer, stained for viability and cell surface antigens, then permeabilized using BD Cytofix/Cytoperm solution (BD Bioscience). Cells were then stained with anti-IFN-γ antibody for 30 min, washed twice in BD Perm/Wash buffer (BD Biosciences), and fixed in FACS fix buffer. For analysis, cells were gated on viable $CD3^-CD56^{bright}CD16^-$, and $CD3^-CD56^{dim}CD16^{+/lo}$ NK cells (Fig. 1A). Percent-specific expression is calculated as the background-adjusted function in the presence or absence of target cell line.

IFN-γ ELISPOT

HCV-specific T cell responses were measured 3 weeks (range: 0–14 weeks) after the NK cell responses were measured (12 weeks; range: 6–17 weeks after estimated time of infection). Ninety six-well polyvinylidene difluoride-backed microtiter plates (Millipore, Bedford, MA, USA) were pre-wet with 35% ethanol (15 μl/well) for 1 min, washed with PBS, and coated overnight at 4 °C with 100 μl/well