Increased number of non-invariant NKT cells and low number of circulating CD1-expressing leukocytes in patients infected with hepatitis C virus

Augmentation du nombre de cellules NKT non invariants et réduction de la circulation de leucocytes exprimant CD1-chez les patients infectés par le virus de l’hépatite C

Abstract. Natural killer T (NKT) cells represent an heterogeneous T cell population involved in host immunity against several microorganisms. They also have important immunoregulatory functions. Studies on circulating levels of NKT cells during HCV infection have been focused on the invariant NKT (iNKT) subset which recognizes the non-classical Ag-presenting molecule CD1d, with little information about the non-invariant NKT (non-iNKT) cell subset. In the present study, we assessed the number of both NKT cells subsets and the surface expression of CD1a, b, c and d isoforms in peripheral blood of 31 HCV-infected patients and 31 ages matched healthy individuals. A significant increase of circulating non-iNKT cells was observed in HCV-infected patients as compared to controls (74 ± 57 cells/L vs 42 ± 16 cells/L respectively, p < 0.0042) with no differences in the iNKT subset. In addition, the percentage of CD1a, CD1c and CD1d-expressing leukocytes was significantly low in patients as compared to controls. These findings suggest that both components, non-iNKT cells and CD1 molecules expression are involved in the control of natural immunity against HCV.

Key words: HCV, invariant NKT cells, non-invariant NKT cells, CD1 system, leukocytes

Résumé. Les cellules natural killer T (NKT) représentent une population hétérogène de cellules T impliquées dans l’immunité de l’hôte contre plusieurs microorganismes. Elles jouent aussi un rôle important dans l’immunorégulation. Les études sur les concentrations circulantes de cellules NKT pendant l’infection par le virus de l’hépatite C (VHC) ont été axées sur le sous-ensemble de cellules invariantes NKT (iNKT), qui reconnaît la molécule CD1 de présentation antigénique non classique, avec peu d’information au sujet du non-iNKT. Dans cette étude, nous avons évalué le nombre de cellules NKT des deux sous-ensembles et l’expression superficielle de CD1a, b, c et des isoformes dans le sang de 31 patients infectés par le VHC et âgés de 31 ans, par rapport à des individus sains de même groupe étaire. Une augmentation significative de la circulation de cellules non-iNKT a été observée chez les patients infectés par rapport aux témoins (74 ± 57 cells/L vs 42 ± 16 cells/L respectivement, p < 0,0042), alors qu’aucune différence n’a été observée dans le sous-ensemble iNKT. En outre, le pourcentage de leucocytes exprimant CD1a, CD1c et CD1d était significativement plus faible chez les patients par rapport aux témoins. Ces résultats suggèrent que les deux composantes, non-iNKT cellules et les molécules d’expression de CD1, sont impliquées dans le contrôle de l’immunité naturelle contre le VHC.

Mots clés : VHC, NKT cellules invariantes, NKT cellules non-invariantes, système CD1, leucocytes
Human NKT cells represent an heterogeneous lymphocyte subpopulation characterized by expressing the CD3+CD56+ phenotype with an important role in tumor surveillance, anti-infectious defenses and maintenance of self-tolerance [1, 2]. A small subset of these cells expresses the semi-invariant T cell receptor (TCR) rearrangement Vα24+Vβ11+chains. This cell subset defined as classical invariant NKT (iNKT) interacts with the monomorphic CD1d molecule which is a family member of the CD1 cell surface glycoproteins involved in lipid antigen presentation to NKT cells [2]. A second subset of CD1-reactive NKT cells known as non-invariant NKT cells (non-iNKT), expresses a diverse TCR receptor and, in contrast to iNKT cells, its effectors functions remains poorly characterized. Among the different isoforms of CD1 able to interact with NKT cells (CD1a, b, c and d) CD1d has been studied most intensively mainly because the identification of potent ligands, such as α-galactosylceramide (α-GalCer) and its analogs [3-6]. Accumulated evidences have made clear that NKT cell-mediated lipid antigen recognition plays an important role in detection and clearance of viral pathogens such as Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV) [3].

NKT cells seem to be involved in the immune response against HCV, a growing worldwide public health problem. However, their specific role in this viral infection is still under discussion [7, 8]. Contrasting data have been reported on the number and role of NKT cells during HCV infection. While some studies have shown no significant differences in the number of circulating iNKT between patients with active HCV infection and healthy donors [9, 10], another study reports a lower numbers of iNKT cells in the blood of HCV positive patients compared to controls [11]. Moreover, there are no reports regarding the possible role of non-iNKT cells in HCV infection.

The aim of this study was to quantify the number of circulating iNKT and non-iNKT cells subsets and to determine the expression of CD1 isoforms in peripheral blood leukocytes from patients chronically infected with HCV.

Methods

Study subjects

A total of 31 patients (19 males and 12 females), age 47±14 years, (means±SD) with diagnosis of HCV chronic infection and without antiviral treatment were selected from the outpatient clinic of the Gastroenterology Unit of the Caracas University Hospital. All patients were repeatedly positive for antibodies against HCV (anti-HCV), positive for HCV RNA in serum and negative for antibodies anti-HBV and anti-HIV. HCV genotype was determined according to Pujol et al. [12]. Virological profile of the 31 HCV-infected patients showed a HCV genotype prevalence of 63% for genotype 1 and 34.6% for genotype 2, with a viral load ranging from 34,711 to 275,000 IU/mL. None of the patients was at end stage of liver disease and they had no evidence of other chronic or autoimmune hepatic diseases. The control group included 31 healthy adult volunteers (15 male and 16 female), mean age 40±12 years’ seronegative for HCV, HBV, and HIV. The protocol study was approved by the Institute’s Bioethical Committee and a written consent was previously obtained from each patient and control included in the protocol.

Flow cytometry analysis

Peripheral blood NKT cells, NK cells, T cells and CD1 molecules were determined by direct staining of 100 μL of whole blood for 20 min at room temperature with an optimal dose of specific monoclonal antibodies anti-CD3/EDC, anti-CD56/PECy5, anti-Vα24/FITC, anti-Vβ11/PE (Immunotech), anti CD1a/FITC, anti-CD1b/RPE, anti-CD1c/FITC (Ancell Corporation) and anti-CD1d/RPE (BD Pharmingen). Erythrocytes were lysated using the automatic Q-prep system (Beckman Coulter; Miami, Fl) and analyzed in a Epics XL Flow Cytometer (Beckman Coulter).

Confocal microscopy analysis of CD1 isoforms in isolated monocytes

Human peripheral blood mononuclear cells (PBMC) were isolated from patients and healthy donors through Ficoll-Hypaque (Pharmacia) density gradient centrifugation. Monocytes were obtained by adherence to plastic culture flasks for 2h at 37°C in an incubator with 5% CO₂ air controlled atmosphere and then harvested by scraping in cold RPMI medium with 2% heat-inactivated fetal calf serum. Monocytes purity was determined by flow cytometry analysis using an anti-human CD14-PE monoclonal antibody (BD Pharmingen). CD1 expression was assessed by staining with specific monoclonal antibodies to CD1 isoforms conjugated to FITC (CD1a, CD1c and PE (CD1b, CD1d). Labeled Monocytes were adhered to a cover glass slide using a cytocentrifuge (Shandon Cytospin II Thermo EC, USA) and then placed in an open experimental chamber (RC-27, Wagner Instrument Corp., USA) and analyzed on a laser scanning confocal microscope (Olympus FV1000, Olympus America, Inc., USA) using a 60X water objective (Olympus Uplan Sapo 60X/NA:1.2). Optical images of cells were generated for each dye after excitation with a 488 nm Argon laser (FITC) and a 559 nm Helium-Neon laser (PE).
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Statistical analysis
A two-tailed paired or unpaired Student’s t-test was used where appropriate to determine the significance of differences between groups, using SPSS 17.0 software (SPSS, Chicago, IL). A P-value of <0.05 was taken as significant.

Results
As shown in figure 1, the four color analysis of the lymphocyte population allowed us to evaluate the total circulating NK cells (CD56+/CD3+), NKT cells (CD3+/CD56+), the iNKT subset (CD56+CD3+/TCRVα24+Vβ11+ + CD56+CD3+/TCRVα24+Vβ11+) and the non-iNKT cell subset (CD56+/CD3+/TCRVα24+Vβ11+), (figure 1A). A significant increase of non-iNKT cell subset was observed in the patients (p ≤ 0.0042) as compared to controls (figure 1C). This increased number of non-iNKT cells in HCV-infected patients did not correlate with their viral load or HCV genotype. No significant differences were found when comparing the levels of NK cells (figure 1B) or iNKT cells (figure 1D) to controls.

Since human CD1 system has different isoforms involved in the presentation of lipid antigens to NKT cells, we evaluated the expression and distribution of the CD1 isoforms (CD1a, CD1b, CD1c and CD1d) through the different sub-populations of peripheral white blood cells using cytometric analysis. With the exception of CD1d which was expressed mainly in monocytes, all other isoforms were detected in lymphocytes, monocytes and granulocytes from both patients and controls. Except for the CD1b isofrom, the percentage of cells expressing the CD1a, c and d isoforms were significantly lower in HCV-infected patients, as compared to controls (figure 2). The cytometric analysis showed that monocytes express the highest percentage of positive cells for all CD1 isoforms. This was further confirmed by confocal microscopy analysis on isolated monocytes from patients and controls (figure 3). Here, we can observe that fluorescence intensity of CD1d is lower in patients’ monocytes than controls, suggesting that in addition to a reduction in the number of CD1d+ monocytes, as showed by flow cytometry, the expression of this molecule is also downregulated in patients with HCV infection (figure 3). Changes in cell surface distribution (fluorescence pattern) of CD1a and CD1b molecules seems to occur in monocytes of HCV-infected patients as compared to controls (figure 3).

![Figure 1](image1.png)

**Figure 1.** Circulating NKT cells numbers in HCV infection. NK cells (CD3+CD56+) and NKT cells (CD3+CD56+) were estimated by gating on lymphocyte cell population. Non invariant (CD3+CD56+Vα24+Vβ11+) and invariant (CD3+CD56+Vα24+Vβ11+ + CD3+CD56+ Vα24+Vβ11+) NKT cells were estimated by gating on total NKT cell population (A). No differences in the number of NK cells were observed between patients and controls (B) whereas a significant increased number of circulating non-iNKT cells was found in HCV-infected patients (C) with no significant variation in the levels of iNKT cells (D).
Discussion

Recent studies on the role of NKT cells against hepatotropic viruses such as HCV have been focused on the iNKT cell subset with few references to the non-iNKT cell subset [13, 14]. Here we show a significant increase in the circulating non-iNKT cell subset of HCV-infected patients with no changes in the iNKT cell subset. No changes in circulating iNKT cells numbers during active HCV infection have been also reported by Karadimitris et al. [9] and by Van der Vliet et al. [10].

Most of the human peripheral blood NKT cells (95-99%) expressed the non-invariant TCR, a cell subset that have been reported to be able to interact with different glycolipids compounds presented by some selected CD1 molecules [3]. To our knowledge very few studies have reported the status of circulating levels of non-iNKT cells in HCV infection. Golden-Mason et al. evaluated the levels of non-iNKT cells in patients with acute HCV infection, reporting that this population was reduced early in the viral infection and did not fluctuate over time [15]. By contrast, our results showed that both total NKT cells and non-iNKT cells were significantly increased in patients chronically infected with HCV, suggesting that this population can augment as the infection becomes chronic. Comparable results have been reported by Weng et al. [16] in Hepatitis B Virus infection. They observed an increased number of non-iNKT lymphocytes in patients with chronic active hepatitis B as compared to asymptomatic carriers and healthy controls. They also found no significant variation in the levels of iNKT cells among the three groups.

Undoubtedly, virus infections are able to activate the heterogeneous NKT cells inducing changes in the different recirculating subsets of these cells which in our study were observed in the non-iNKT subset and according with other studies they have potent cytotoxic properties and produce Th1 cytokines [3]. These CD1 (CD1a, CD1b or CD1c)-restricted T cells appears to have a broad range of functions, including most of the effector capabilities of MHC Class I and Class II-restricted T cells. They may express CD4, CD8 or can be CD4/CD8 double negative (DN) [3]. Further analyses of these cells are important to carry out in order to clarify their role in HCV infection.

CD1 system participates in the innate immune response through the presentation of glycolipid antigens to the NKT cells, which can influence a wide array of immune responses including anti-microbial immunity [17]. In this study we observed a significant low frequency of leukocytes expressing the different CD1 isoforms in HCV-infected patients which suggest that HCV infection may down regulate the expression of CD1 molecules in order to block their recognition. In this sense, previous studies of our group have shown that HCV can interact with different leukocyte populations affecting some cellular functions [18-20].

![Figure 2](image-url)

**Figure 2.** CD1 expression in PBL of HCV-infected patients and healthy controls. CD1 expression was determined by flow cytometry in peripheral blood lymphocytes (lymph), monocytes (mono) and granulocytes (gran) using specific mAbs conjugate to FITC (CD1a, CD1c) and PE (CD1b, CD1d). Data are expressed as percentage of positive cells.
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Recently it has been stated that certain viral infections can down regulate CD1d expression [21]. In the case of the Kaposi sarcoma-associated herpes virus and herpes simplex virus, this effect is exerted by two viral modulators of immune recognition proteins which ubiquinates the cytoplasmatic tail of CD1d and thereby accelerates its endocytosis and lysosomal targeting [22, 23]. Also, Herpes simplex virus infection is associated to a recycling defect of CD1 from the endosome to the cell surface due to entrapment of the protein in the late endosome. Finally, HIV infection increases the internalization of CD1d from the cell surface and holds it in the trans-Golgi network [24].

In our study we observed down regulation not only of CD1d but also of CD1a and CD1c; suggesting that other subsets of NKT cells different to invariant NKT cells may be affected as effectors in the immune response against HCV. These CD1 isoforms are responsible for glycolipid-antigen presentation to different subsets of NKT cells; therefore they can be targets of the modulatory mechanisms of the virus in order to evade its recognition.

Figure 3. Confocal microscopy analysis of CD1 isoforms. Expression of CD1a, CD1b, CD1c and CD1d in peripheral blood monocytes isolated from HCV-infected patients and controls (purity > 90%) was assessed using specific mAbs and confocal microscopy analysis. Non-specific binding was evaluated using the corresponding mouse IgG isotype.
Even though no virally derived CD1 ligands have been yet identified, a recent report shows that CD1c presents an N-terminally acylated lipopeptide similar in sequence to HIV nuclear envelope factor (Nef) [25]. This finding supports the hypothesis that cellular lipidation of viral proteins may generate antigens presented by CD1 [26]. Recently, biochemical and morphological characterization of HCV particles has demonstrated the unique lipid composition of the virus particle which is composed of very low and low density lipoproteins, with cholesteryl esters accounting for almost half of the total HCV lipids [27]. Further studies are needed to determine the possible role of HCV lipids constituents as potential antigens targets presented by the CD1 system and their association with the NKT function in HCV infection. In addition, the possible role that HCV infection may have in the regulation of CD1 expression is a matter that requires more investigation.

In summary, we have found that non-invariant NKT cells subset are significantly increased in HCV-infected individuals, suggesting an important role for these cells in the control of this viral infection. In addition, the significant low number of leukocytes bearing the CD1 molecules observed in HCV-infected patients may represent a condition that contribute to the chronic state of HCV infection.

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Conflicts of interest: none.

References


