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MINIREVIEWS

- 1043** Hepatitis B in patients with hematological diseases: An update
Coluccio C, Begini P, Marzano A, Pellicelli A, Imperatrice B, Anania G, Delle Fave G, Marignani M
- 1054** Hepatitis B in renal transplant patients
Marinaki S, Kolovou K, Sakellariou S, Boletis JN, Delladetsima IK

ORIGINAL ARTICLE

Retrospective Cohort Study

- 1064** Real-world efficacy of daclatasvir and asunaprevir with respect to resistance-associated substitutions
Fujii H, Umemura A, Nishikawa T, Yamaguchi K, Moriguchi M, Nakamura H, Yasui K, Minami M, Tanaka S, Ishikawa H, Kimura H, Takami S, Nagao Y, Shima T, Itoh Y

Observational Study

- 1073** CD4⁺ T cells and natural killer cells: Biomarkers for hepatic fibrosis in human immunodeficiency virus/hepatitis C virus-coinfected patients
Laufer N, Ojeda D, Polo ML, Martinez A, Pérez H, Turk G, Cahn P, Zwirner NW, Quarleri J

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Observational Study

CD4⁺ T cells and natural killer cells: Biomarkers for hepatic fibrosis in human immunodeficiency virus/hepatitis C virus-coinfected patients

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Abstract**AIM**

To characterize peripheral blood natural killer (NK) cells phenotypes by flow cytometry as potential biomarker of liver fibrosis in human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfecting patients.

METHODS

Peripheral mononuclear cells from 24 HIV/HCV (HBV

negative) coinfecting and 5 HIV/HCV/HBV seronegative individuals were evaluated. HIV/HCV coinfecting patients were divided in to groups: G1, patients with METAVIR F0-F2 and G2, patients with METAVIR F3-F4. NK surface cell staining was performed with: Anti-CD3(APC/Cy7), anti-CD56(PE/Cy5), anti-CD57(APC), anti-CD25(PE), anti-CD69(FITC), anti-NKp30(PE), anti-NKp46(PE/Cy7), anti-NKG2D(APC), anti-DNAM(FITC); anti-CD62L (PE/Cy7), anti-CCR7(PE), anti-TRAIL(PE), anti-FasL(PE), anti CD94(FITC). Flow cytometry data acquisition was performed on BD FACSCanto, analyzed using FlowJo software. Frequency of fluorescence was analyzed for all single markers. Clinical records were reviewed, and epidemiological and clinical data were obtained.

RESULTS

Samples from 11 patients were included in G1 and from 13 in G2. All patients were on ARV, with undetectable HIV viral load. Liver fibrosis was evaluated by transient elastography in 90% of the patients and with biopsy in 10% of the patients. Mean HCV viral load was ($6.18 \pm 0.7 \log_{10}$). Even though, no major significant differences were observed between G1 and G2 regarding NK surface markers, it was found that patients with higher liver fibrosis presented statistically lower percentage of NK cells than individual with low to mild fibrosis and healthy controls (G2: $5.4\% \pm 2.3\%$, G1: $12.6\% \pm 8.2\%$, $P = 0.002$ and healthy controls $12.2\% \pm 2.7\%$, $P = 0.008$). It was also found that individuals with higher liver fibrosis presented lower CD4 LT count than those from G1 (G2: 521 ± 312 cells/ μ L, G1: 770 ± 205 cells/ μ L; $P = 0.035$).

CONCLUSION

Higher levels of liver fibrosis were associated with lower percentage of NK cells and LTCD4⁺ count; and they may serve as noninvasive biomarkers of liver damage.

Key words: CD4⁺ T cell; Human immunodeficiency virus/hepatitis C virus-coinfection; Fibrosis; Biomarker; Natural killer cells

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Core tip: Approximately 2.3 million individuals with human immunodeficiency virus are coinfecting with hepatitis C virus (HCV). The high cost of HCV treatment restricts its use. It is crucial to identify patients with advanced liver fibrosis with an urgent need of treatment. The aim of this study was to identify natural killer (NK) phenotypes as a biomarker for liver fibrosis. We observed that those subjects with higher fibrosis are those with lower percentage of NK cells and also with lower LTCD4⁺ count. These constitute two simple parameters that might be performed in a routine laboratory test and used in clinical practice as biomarkers for liver fibrosis.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection affects 115 million individuals worldwide and is a common cause of chronic hepatitis, which may eventually progress to cirrhosis and hepatocellular carcinoma^[1]; whereas currently 36.9 million people are living with human immunodeficiency virus (HIV)/aids^[2]. Because of overlapping pathways of transmission, approximately 2.3 million individuals worldwide are estimated to be coinfecting with both viruses^[3]. Direct antiviral agents (DAA) are a major development in the treatment of HCV infection, with cure rates higher than 90%^[4]. However, the high cost of DAA regimens and competing public health priorities have prompted a worldwide discussion whether all patients should have access to the new therapies without restriction. In many countries, new DAA regimens are therefore reserved for patients with advanced fibrosis or cirrhosis^[5,6].

Liver fibrosis is a response to a wound-healing process triggered by various types of chronic liver injuries, among them HCV infection^[7]. Liver fibrosis is well characterized by abnormal accumulation of extracellular matrix, and hepatic stellate cells (HSCs) are considered to be the major type of cells responsible for liver fibrosis. Such profibrotic role might be down-regulated by natural killer (NK) cells either directly through induction of HSC apoptosis or indirectly *via* production of IFN- γ . Increased peripheral NK cell-mediated cytotoxicity has been associated with less liver fibrosis during HCV infection and likely reflects this mechanism^[7]. HIV infection *per se* has a strong suppressive effect on anti-HCV activity of NK cells^[8].

NK cells are lymphoid cells that are primary responders to microbial infections and tumor cells^[9]. Phenotypically, NK cells are defined as CD3⁺CD56⁺ cells with variable expression of CD16, depending on cell subpopulation of NK cells. They comprise approximately 5%-20% of peripheral lymphoid cells, but up to 30%-50% of intrahepatic lymphoid cells. NK cell activation is regulated by cell surface receptors that become engaged by cognate ligands expressed on target cells by cytokines, and by Toll-like receptors (TLRs)^[9-11].

Different techniques to assess liver fibrosis have been developed, from liver biopsy (gold standard) to non-invasive studies (transient liver elastography; patented and nonpatented biomarkers - FIB4, Fibro-Test, APRI, *etc*). Liver biopsy is invasive and has risk of complications^[12]. In addition, liver biopsy may be limited by the size of the specimen obtained as well as sampling,

Table 1 Fluorochrome-conjugated antibodies panels

Antibody	Fluorochrome	Clone	Provider
All panels			
Anti-CD3	APC/Cy7	SK7	BioLegend
Anti-CD56	PE/Cy5	679.1Mc7	Beckman Coulter
Panel 1			
Anti-CD57	APC	HNK-1	BioLegend
Anti-CD25	PE	BC96	BioLegend
Anti-CD69	FITC	FN50	BioLegend
Panel 2			
Anti-NKp30	PE	P30-15	Biolegend
Anti-NKp46	PE/Cy7	9E2	Biolegend
Anti-NKG2D	APC	1D11	Biolegend
Anti-DNAM	FITC	TX25	Biolegend
Panel 3			
Anti-CD62L	PE/Cy7	DREG-56	Biolegend
Anti-CCR7	PE	G043H7	Biolegend
Panel 4			
Anti-TRAIL	PE	S35-934	BD Bioscience
Panel 5			
Anti-FasL	PE	NOK-1	Biolegend
Anti CD94	FITC	DX22	Biolegend

BioLegend, San Diego, California United States. BD Bioscience, San Jose, California, United States. NK: Natural killer.

intraobserver, and interobserver variability^[13]. On the other hand, there are many unresolved issues regarding the accuracy (especially in HIV/HCV-coinfected patients) of noninvasive studies^[14], and in low-resource countries there is an important barrier to access to these methods (in particular liver elastography and patented biomarkers).

The identification of noninvasive liver fibrosis biomarkers is still an open research area. In this context, we reasoned that the study of the phenotype of peripheral blood cells may unravel interesting clues towards the identification of such biomarkers. Some evidence indicates that the characteristics of the immune cells, including NK cells, observed in peripheral blood are similar to those seen in liver with relatively lower levels of magnitude. Accordingly, the aim of this study was to characterize peripheral blood NK cell phenotypes by flow cytometry as potential biomarker for liver fibrosis in patients chronically coinfected with hepatitis C and HIV.

MATERIALS AND METHODS

Study cohort

Informed consent was obtained from each subject. The study protocol is in line with the ethical guidelines of the Declaration of Helsinki and was approved by the ethics review committee of Fundación Huésped (Buenos Aires, Argentina).

Cryopreserved peripheral blood mononuclear cells (PBMC) from 24 HIV/HCV-coinfected individuals and 5 HIV/HCV-seronegative individuals (healthy controls, HC) were used in this study. HIV/HCV-coinfected patients and healthy control individuals enrolled in this study were not acutely or chronically infected with

HBV; they denied current use of recreational drugs or alcohol intake. HIV/HCV-coinfected patients were divided into two groups based on their level of liver fibrosis (group 1: Patients with METAVIR score F0 to F2 on liver biopsy or transient elastography - FibroScan® -; and group 2: Patients with METAVIR score F3-F4). Hepatic fibrosis was evaluated by liver biopsy in 10% of patients and by transient hepatic elastography in 90% of patients. All healthy control individuals presented F0-F1 fibrosis according to transient liver elastography (less than 5 kPa). Clinical records were reviewed, and epidemiological and clinical data were obtained.

Multicolor flow cytometry

Cryopreserved PBMC were thawed and stained with fluorochrome-conjugated antibodies distributed in five different panels (depending on PBMC availability) to evaluate expression of different markers on NK cells detailed in Table 1. Staining was performed for 30 min at 4 °C. Samples were washed, fixed in 1% paraformaldehyde and acquired in a FACS Canto flow cytometer (BD Biosciences). Data were analyzed using the FlowJo software (TreeStar, Ashland, Oregon, United States). NK cell populations were defined according to the corresponding isotype control.

Plasma viral load levels (Abbott RealTime HIV-1 RNA version 3; Abbott Molecular, Inc., Des Plaines, IL, United States) were assessed in HIV-infected subjects and CD4⁺ T-cell counts (flow cytometry double platform, BD FACSCanto; BD Biosciences, San Diego/California, United States) were assessed in HIV and HIV-negative individuals.

Statistical analysis

For categorical variables, both χ^2 and Fisher's exact test were applied. For continuous variables, the nonparametric Kruskal-Wallis and Mann-Whitney test were used. Area under the receiving operating curve (ROC) was used to calculate the cut-off point in NK cell percentage with the best sensitivity of high liver fibrosis. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 19.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patient characteristics at the time of liver fibrosis assessment are shown in Table 2. Individuals from Group 1 ($n = 11$, 46%) presented low to mild liver fibrosis (METAVIR F0-F2) whereas patients included in Group 2 ($n = 13$, 54%) had severe fibrosis (METAVIR F3-F4). Forty percent of patients had previously received HCV treatment with pegylated interferon and ribavirin (with no differences between groups); a median of 6.25 ± 1.48 years before sample collection; none of them achieved sustained virological response. The mean age was 46.9 years (± 8.4); 83% were male. Patients from group 2 were older than those with lower METAVIR score ($P = 0.028$). No differences were

Table 2 Characteristics of the population and divided according the level of fibrosis

Characteristics	Group 1 <i>n</i> = 11	Group 2 <i>n</i> = 13	Control <i>n</i> = 5	<i>P</i> value
Age (yr) ¹	46.3 (3.9)	52.2 (4.5)	31 (4.8)	0.02
Male/female	9/2	11/2	3/2	0.85
CD4 cell count ¹	770 (205)	521 (312)	910 (251)	0.03
CD8 cell count ¹	1079 (475)	657 (339)	NA	0.24
METAVIR F0-F2	100%	0	100%	NC
METAVIR F3-F4	0	100%	0	NC
TGP ²	79.7 (74.2)	99 (71.4)	NA	0.41
Time of known HIV infection ¹	18 (6.52)	17.3 (3.8)	NC	0.60
Time of known HCV infection ¹	14.3 (7.0)	13.8 (4.6)	NC	0.65

¹mean \pm SD. Beckman Coulter, Marseille, France. *P* values, correspond to comparison between group 1 and 2. NA: Not available; NC: Not correspond; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus.

found between groups regarding gender or mean time of known HIV and HCV infection. The mean HCV viral load was 6.18 ± 0.70 log, with no differences between the two groups (G1: 6.54 ± 0.24 ; G2: 6.18 ± 0.7). HCV genotype 1 was identified in 90% of the patients, the rest presented infection by genotype 3. All patients were on antiretroviral treatment with undetectable HIV viral load, with no differences in the time on ARV therapy between groups. Patients with higher fibrosis presented lower CD4⁺ T cell count (521 ± 312 cells/ μ L) than those from group 1 (770 ± 205 cells/ μ L, *P* = 0.035) There was no difference in the CD4⁺ T cell count between group 1 and healthy controls (*P* = 0.49).

Regarding NK cells, a lower percentage was found in samples from patients of group 2 ($5.4\% \pm 2.3\%$) compared both with patients from group 1 ($12.6\% \pm 8.2\%$, *P* = 0.002) and healthy controls ($12.2\% \pm 2.7\%$, 0.008) (Figures 1 and 2). With ROC curve analysis a cut-off of a NK cell percentage lower than 6.6% was determined to have 90% sensitivity and 77% specificity to predict the presence of METAVIR F3-F4 (Figure 3).

The percentage of CD56^{bright} NK cells (G1: $11.7\% \pm 8.0\%$, G2: $7.1\% \pm 4.0\%$, HC: $6.8\% \pm 3.6\%$) and CD56^{dim} NK cells (G1: $88.2\% \pm 7.6\%$, G2: $73.7\% \pm 40.1\%$, HC: $92.9\% \pm 3.6\%$) did not present differences among the three groups studied.

As the function of NK is regulated by an array of activating and inhibitory receptors, we also evaluated the NK cell activating receptors^[15] NKp46 (CD335), NKp30 (CD337), NKG2D (CD314) and DNAM (CD226), the activation markers CD69 and CD25, and other molecules involved in NK cell effector functions, terminal differentiation and cytotoxicity such as CD94, TRAIL, CD57^[16], Fas-L (CD178), CCR7 (CD197) and CD62L.

When compared with healthy controls, samples from patients included in group 2 presented a higher frequency of CD56^{bright} DNAM-1⁺ NK cells ($76.2\% \pm 18.5\%$ vs $4.6\% \pm 8.5\%$, *P* = 0.008) and CD56^{dim} DNAM-1⁺ NK cells ($42\% \pm 29\%$ vs $6.0\% \pm 8.1\%$, *P* = 0.018). The same

differences were observed between group 1 and healthy controls, both in the percentage of CD56^{bright} DNAM-1⁺ NK cells ($71.2\% \pm 23\%$ vs $4.6\% \pm 8.5\%$, *P* = 0.003) and in the percentage of CD56^{dim} DNAM-1⁺ NK cells ($41\% \pm 28\%$ vs $6.0\% \pm 8.1\%$, *P* = 0.013).

Additionally, samples from group 1 exhibited higher percentage of CD56^{bright} CD25⁺ NK cells ($53.1\% \pm 16.6\%$ vs $19.4\% \pm 18.9\%$, *P* = 0.029) and CD56^{dim} CD25⁺ NK cells ($28.3\% \pm 10.2\%$ vs $7.1\% \pm 5.6\%$, *P* = 0.001) than healthy controls. These results show the possible consequence of a higher activation degree in NK cells from subjects with chronic infection. Of note, there were no differences in the frequency of these NK cells subsets between group 1 and 2. Moreover, no differences were observed in the other activator molecules evaluated (NKp46, NKp30, NKG2D, CD69) neither between group 1 and 2 nor between controls and HCV-infected subjects.

No differences in surface expression of CD94 were observed between the 3 groups. The frequency of these molecules was very high in CD56^{bright} NK cells in all the samples evaluated (G1: $77.1\% \pm 28.2\%$, G2: $91.7\% \pm 1.2\%$, controls: $77.4\% \pm 36.7\%$), whereas in CD56^{dim} NK cells this molecule was stained in less than 50% (G1: $48.4\% \pm 21.4\%$, G2: $36.9\% \pm 19.7\%$, controls: $31.6\% \pm 16.9\%$).

The frequency of CD56^{dim} TRAIL⁺ NK cells was higher in samples from group 2 than those from group 1 ($29.4\% \pm 31.7\%$ vs $7.5\% \pm 3.1\%$, *P* = 0.04), while no differences were observed between coinfecting patients and healthy controls.

Nevertheless, the percentage of CD56^{dim} FasL⁺ NK cells was lower in samples from HCV/HIV-coinfecting patients (G1: $27.2\% \pm 19.8\%$, *P* = 0.001; G2: $36.9\% \pm 19.7\%$, *P* = 0.01) than those from healthy controls ($69.3\% \pm 18.2\%$), without detecting differences between groups 1 and 2.

In addition, there was a trend towards a higher percentage of CD56^{dim} CCR7⁺ NK cells in samples from patients with advanced fibrosis than in samples from patients with lower fibrosis (G2: $56.4\% \pm 36.2\%$ vs G1: $24.4\% \pm 14.6\%$; *P* = 0.05). Regarding the CD62L expression, there were no differences in CD56^{bright} NK cells between groups (G1: $61.8\% \pm 24.9\%$; G2: $87\% \pm 15.1\%$; *P* = 0.09).

DISCUSSION

In this study we found that patients with advanced fibrosis presented lower LT CD4⁺ cell counts than subjects with low to mild fibrosis. All the patients were on successful antiretroviral treatment. Even though there are controversial data whether the presence of HCV is a factor that alters LT CD4 recovery with ARV, it can be hypothesized that patients with higher chances to develop liver fibrosis are those with lower LT CD4⁺ cell recovery after HIV treatment. Such a poor HAART-mediated LT CD4⁺ cell recovery may

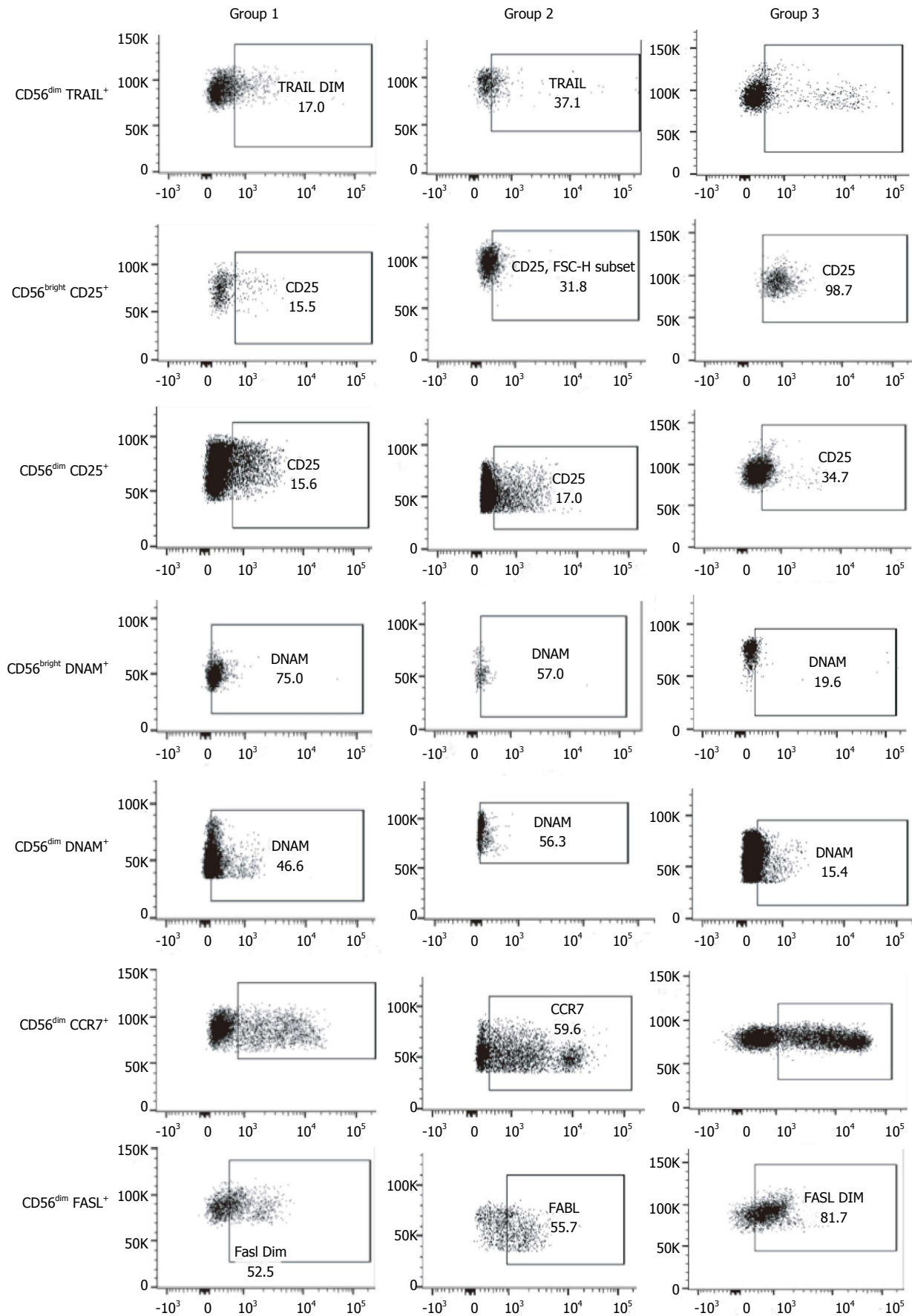


Figure 1 Representative dot plots of each of groups evaluated.

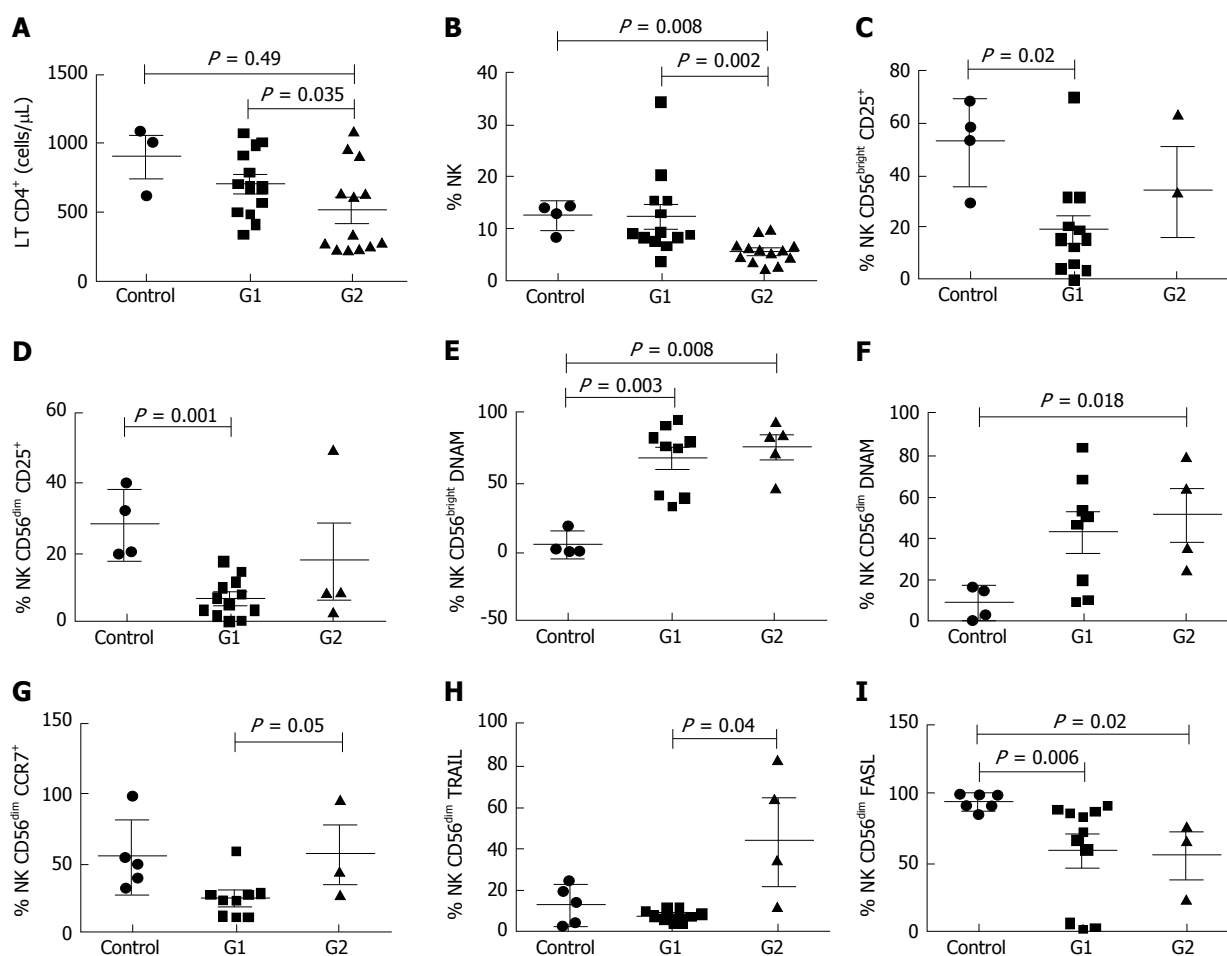


Figure 2 Differences between groups 1, 2 and controls regarding. A: Total CD4 cell count; B: Percent of NK cells; C: Percent NK CD56^{bright} CD25⁺; D: Percent CD56^{dim} CD25⁺ NK cells; E: Percent CD56^{bright} DNAM⁺ NK cells; F: Percent CD56^{dim} DNAM⁺ NK cells; G: Percent CD56^{dim} CCR7⁺ NK cells; H: Percent CD56^{dim} TRAIL⁺ NK cells; I: Percent CD56^{dim} FasL⁺ NK cells. The P values are shown in each graphic and the line below the P value connects the two groups compared. NK: Natural killer.

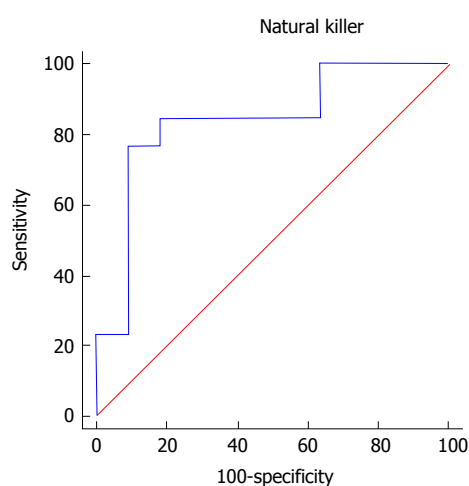


Figure 3 Area under the receiver operating characteristic curve, to evaluate the performance of natural killer cell % with liver fibrosis as the state variable.

contribute to an impaired stimulation of NK cells, and consequently a diminished anti-fibrotic activity by their action on hepatic stellate cells, favoring an accelerated liver fibrosis progression in HIV/HCV patients^[17]. Yi *et al*^[18] and other groups have observed

that NK cells negatively regulated liver fibrosis. NK cells isolated from HCV-infected patients efficiently induced apoptosis of activated HSCs in TRAIL-, FasL-, and NKG2D-dependent manners^[19]. NKp46^{high} NK cell subset potentially suppresses HCV replication and HCV-associated liver damage, leading to amelioration of liver fibrosis.

It has been described that HIV/HCV coinfection can modulate the peripheral NK phenotype^[20]. In our study, we also observed differences in the NK phenotype particularly between control and HIV/HCV-coinfected patients which resemble those reported previously^[21,22]. We found a lower percentage of CD56^{dim} FasL⁺ NK cells in HCV/HIV-coinfected patients compared to healthy controls. This finding could reflect a lower NK cell capacity to exert cytotoxic activity in patients with chronic HIV and HCV infection compared to non-infected individuals that could ultimately lead to a decreased capacity to regulate HSC.

Regarding HIV/HCV-coinfected individuals, no differences were observed in NK cell phenotypes according to the different degrees of liver fibrosis. Nevertheless, we could observe a statistically significant difference in the percentage of peripheral

blood NK cells in patients with high scores compared to patients with low liver fibrosis. Patients with advanced fibrosis have lower percentage of NK cells than those with low fibrosis scores. Moreover, we observed that a percentage of NK cells lower than 6.6% had 90% sensitivity and 77% specificity to predict the presence of advanced fibrosis (METAVIR F3-F4). This observation could indicate, for the first time, that the evaluation of the NK cells compartment is a potential biomarker for fibrosis staging in HIV/HCV-coinfected patients.

In the era of direct antiviral agents with high efficacy for the treatment of chronic HCV, one of the main treatment access barriers for many patients is the high cost of these drugs, and where these barriers exist the assessment of liver fibrosis is mandatory to ensure treatment access. In this study, we have observed that those subjects with higher fibrosis are those with lower absolute count both of LT CD4⁺ and lower percentage of NK cells. Although additional research is needed to confirm our findings, the evaluation of these two parameters that can be performed in a routine laboratory test may be helpful in improving the available noninvasive methods for liver fibrosis staging.

COMMENTS

Background

Different techniques to assess liver fibrosis have been developed, from liver biopsy (gold standard) to non-invasive studies (transient liver elastography; patented and non-patented biomarkers - FIB4, FibroTest, APRI, *etc.*). Liver biopsy is invasive and has risk of complications. In addition, liver biopsy may be limited by the size of the specimen obtained as well as sampling, intra and inter-observer variability. On the other hand, there are many unresolved issues regarding the accuracy [especially in human immunodeficiency virus (HIV)/ hepatitis C virus (HCV)-coinfected patients] of noninvasive studies, and in low-resource countries there is an important barrier to access to these methods (in particular liver elastography and patented biomarkers).

Research frontiers

The identification of non-invasive liver fibrosis biomarkers is still an open research area. In this context, the authors reasoned that the study of the phenotype of peripheral blood cells may unravel interesting clues towards the identification of such biomarkers. Some evidence indicates that the characteristics of the immune cells, including natural killer (NK) cells, observed in peripheral blood are similar to those seen in liver with relatively lower levels of magnitude. Accordingly, the aim of this study was to characterize peripheral blood NK cell phenotypes by flow cytometry as potential biomarker for liver fibrosis in patients chronically coinfected with hepatitis C and HIV.

Innovations and breakthroughs

In the era of direct antiviral agents with high efficacy for the treatment of chronic HCV, one of the main treatment access barriers for many patients is the high cost of these drugs, and where these barriers exist, the assessment of liver fibrosis is mandatory to ensure treatment access. In this study, the authors have observed that those subjects with higher fibrosis are those with lower absolute count both of LT CD4⁺ and lower percentage of NK cells. Although additional research is needed to confirm their findings, the evaluation of these two parameters that can be performed in a routine laboratory test may be helpful in improving the available noninvasive methods for liver fibrosis staging.

Applications

The data in this study suggested that LTCD4 and NK cells could be used as potential non-invasive biomarkers of the level of liver fibrosis in HIV-HCV

coinfected patients. These parameters could improve the accuracy of the available non-invasive methods to measure liver fibrosis.

Terminology

NK cells, and CD4 T lymphocytes (LTCD4) are involved in the immunological control of hepatic stellate cells that are the responsible of liver fibrosis development.

Peer-review

The identification of noninvasive liver fibrosis biomarkers is still an open research area. NK cells and LTCD4⁺ count; are two simple parameters, that might be perform in a routine laboratory test and may serve as noninvasive biomarkers of liver fibrosis, identifying patients in need for HCV therapy in the short term.

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