Gender as a factor affecting the NK cell activity in patients successfully treated for chronic hepatitis C with DAA

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Abstract

Chronic hepatitis C (CHC) affects the activity of NK cells, but successful interferon-free treatment partially restores it. The goal of the study was to assess whether gender influences those alterations. We examined 21 women after menopause and 24 men with CHC treated with directly acting antivirals (DAA), and 33 healthy volunteers. With flow cytometry, we analysed KIR2DS4, NKG2D, NKp30, KIR2DL2/DL3, NKG2A, TRAIL and granzyme B on the surface of NK cells, simultaneously we checked serum CXCL10 with ELISA. Overall, patients with CHC had higher expression of KIR2DS4, NKG2A, NKp30 and a lower percentage of NK cells among lymphocytes than the control group. After the treatment KIR2DS4, KIR2DL2/DL3 and NKG2A, TRAIL NKp30 on NK cells decreased, while the percentage of NK cells, expression of granzyme B and NKG2D increased. Serum CXCL10 was elevated before the treatment and dropped afterwards. We observed differences between genders in the expression of KIR2DL2/DL3 (higher in female) and NKp30 (elevated in men) comparing CHC/control groups. After the treatment of KIR2DL2/DL3, NKp30 and CXCL10 dropped only in female while granzyme B increased in male. In conclusion, the response of NK cells among men and women in post-menopausal age with CHC differs. Our research may lead to more studies on different nature of female and male immune systems in the context of HCV infection and treatment.

Introduction

Natural killer cells are considered a crucial element of the innate immune response against viral infections. We divide their effector functions into two main categories. First, they present direct cytotoxicity either by releasing cytotoxic granules containing perforin and granzymes or inducing death receptor-mediated apoptosis by expressing Fas ligand or TNF-related apoptosis-inducing ligand (TRAIL)¹. Second, NK cells produce pro-inflammatory and immunosuppressive cytokines as well as chemokines².

To activate their effector functions, NK cells do not possess antigen-specific receptors nor require antigen presentation. The process is regulated by several inhibitory or activating surface receptors and their ligands on target cells. On healthy cells, MHC class I molecules prevent mobilisation and cytotoxicity of NK cells via inhibitory KIR receptors. Virus-infected cells usually decrease MHC class I expression and increase the presentation of stimulating ligands³.

Chronic hepatitis C (CHC) does not simply lead to increased activation of NK cells but produces a dysregulation of their functions instead⁴. Expression of particular activating and inhibiting receptors on NK cells is distorted; nevertheless, the involved authors show various results of their study, as presented in table 1.

	NK effector function	Chronic	After DAA treatment
KIR2DS4 (CD158i)	Activating receptor	↔ ^{5 6}	?
NKG2D	Activating receptor	↑ ⁷ ↔ ⁸ ↓ ^{9 10 11}	↔ ^{12 8}
NKp30	Activating receptor	↑ ^{12 13 14} ↓ ^{15 16}	↓ ^{12 8 14}
KIR2DL2/DL3 (CD158b)	Inhibiting receptor	↑ ^{9 16 14} ↔ ⁵	↓ ¹⁴
NKG2A	Inhibiting receptor	↑ ^{5 8 15 17 14}	↓ ^{8 14}
TRAIL	Direct cytotoxicity	↑ ^{12 17}	↓ ^{12 8}
Granzyme B	Cytotoxic granules	↑ ¹⁸	↑ - combined therapy (with IFN) ¹⁹ ↓ ¹⁸
CXCL10	Chemokine	↑ ⁸	↓ ⁸

Table 1 Parameters of NK cells examined in the current study, assessed by other authors. Arrows indicate alterations observed among chronically HCV-infected patients and changes after the successful therapy with direct-acting antivirals (DAA).

Female and male immune systems differ in many aspects. Causes of sexual dimorphism are both hormonal and genetic. Females develop enhanced innate and adaptive immune responses comparing to males, which makes them less predisposed to various infections and malignancies, but more susceptible to autoimmune diseases²⁰. Regarding NK cells, males have a higher count of NK cells, NK56dim cells proportion and higher expression of CD57²¹. In women of reproductive age estrogens inhibit NK cells cytotoxicity at the ovulatory phase²², but in older age women have more vigorous cytotoxic activity than men²³. Among chronically HBV-infected patients, intrahepatic NK cells have higher degranulation activity in women and correlate with estradiol level²⁴.

Gender disparity also has an impact on prognosis in CHC. Immunological differences combine with hepatoprotective effects of estradiol and estrogen²⁵. As a result, in HCV-associated cirrhosis male patients with hepatocellular carcinoma (HCC) have a worse outcome than women²⁶. Short HCV infections treated with interferon-based therapies had higher chances of success in female²⁷. Among HCV-infected patients treated with direct-acting antivirals, HCC recurrence and occurrence rate is higher among men²⁸.

The goal of the current study was to investigate whether gender influences changes observed in NK cells of chronically HCV-infected patients cured with the novel, interferon-free therapies.

Materials And Methods

We collected a study group from among patients treated at the Department of Infectious Diseases, Hepatology and Acquired Immunodeficiencies of Poznan University of Medical Sciences. It consisted of 45 patients with CHC genotype 1b qualified to DAA treatment, 21 female and 24 male with different stages of liver fibrosis. To eliminate the impact of rapid hormonal changes in the course of the menstrual cycle, we decided to qualify only female after menopause. Exclusion criteria were: the presence of HCC,

HIV co-infection, presence of HBsAg, administering of any immunomodulatory or immunosuppressive drugs.

We tested the subjects up to 7 days before the treatment (T0) and twice after the completed regimen – an average of 200 days after the first dose (T1) and 167 days later (T2). Twenty-two of patients were treated with sofosbuvir/ledipasvir (among them 16 regiments with ribavirin), 7 received ombitasvir/paritaprevir/ritonavir/dasabuvir, four grazoprevir/elbasvir, eight sofosbuvir/velpatasvir and four got glecaprevir/pibrentasvir. We applied the regiments according to the polish recommendations²⁹. All the patients completed the therapy and achieved a sustained virological response (SVR). Then, we enrolled a control group that consisted of 33 volunteers without HCV infection, 22 men and 11 women in post-menopausal age.

We measured liver stiffness with transient elastography technique, using FibroScan Compact device (Echosens, Paris, France) in compliance with the manufacturer's instructions³⁰.

Cell immunophenotyping was performed by flow cytometry with the use of direct fluorescence method, which we described in our previous study³¹. Briefly, the appropriate monoclonal antibodies fluorochrome-labelled (described in Table 2) were added to the cytometric tubes at a rate of 5 ml each. 100 ml of peripheral blood mixed with EDTA were added to the test tubes. Samples were mixed gently on vortex and incubated for 15 min at room temperature protected from light. After this time, 500 ml of erythrocyte lysis buffer (Becton Dickinson) were added to the test tubes and incubated for 10 min. Lysis was stopped by adding PBS solution (phosphate-buffered saline) and centrifuged. This step was repeated. Granzyme B was studied intracellular after additional permeabilisation of cells with Perm/Wash buffer (BD Biosciences). All obtained cell pellets were resuspended in PBS buffer and were acquired using a FACS Canto flow cytometer (Becton Dickinson). The obtained results were analysed with the FACS Diva software (Becton Dickinson), integrated with the cytometer. For each examined antibody percent of positive cells and Mean Fluorescence Intensity (MFI) was determined.

Table 2. Antibodies used in the study for cell immunophenotyping.

Antibody	Fluorochrome	Clone	Source
CD158b (KIR2DL2/DL3)	PE	DX27	Miltenyi Biotec
CD158i (KIR2DS4)	PE	JJC11.6	Miltenyi Biotec
CD159a (NKG2A)	PE	REA110	Miltenyi Biotec
CD314 (NKG2D)	PE	BAT221	Miltenyi Biotec
CD337 (NKp30)	PE	AF29-4D12	Miltenyi Biotec
CD253 (TRAIL)	PE	RIK-2	BD Pharmingen™
Granzyme B	PE	GB11	BD Pharmingen™

Concentration of human CXCL10/IP-10 in serum was measured using appropriate DuoSet® Immunoassay Development kits (R&D Systems, Minneapolis, USA), according to manufacturer's instructions.

The statistical analysis was conducted using STATISTICA software version 13.0 (StatSoft, Cracow, Poland). Since in Shapiro-Wilk test we evaluated most of our variables to not be normally distributed, we were using non-parametrical tests. We analysed dependent variables with Wilcoxon signed-rank test and non-dependent ones with Mann-Whitney U test. A p-value ≤ 0.05 was considered statistically significant

Results

Among the patients qualified to the study, 66,7% had radiological and biochemical indicators of liver cirrhosis, confirmed in FibroScan. There were no significant differences between genders in assessed parameters of the liver function nor basic biochemical and morphological parameters, presented in table 3. After the completed treatment hepatic stiffness, alanine transaminase (ALT) aspartate transaminase (AST) and total bilirubin level decreased significantly ($p=0,002$, $0,00002$, $0,00002$ and $0,007$, respectively) while platelet count (PLT) and white blood cells count (WBC) increased ($p=0,0004$ and $0,00002$, respectively). Observed changes were similar among genders.

Table 3 Characteristic of the study group and the control group. ALT - alanine transaminase, AST - aspartate transaminase. In Mann-Whitney U test there are no significant differences between female and male in presented parameters. Data are median (interquartile range).

	CONTROL	T0	T1	T2
Total number	33	45	-	29
Female	11	21	-	11
Male	22	24	-	18
Age, years	47 (31-65)	62 (56-68)	-	62 (51-70)
Female	52	66	-	66
Male	38	60	-	61
Estimated time of infection, years	-	37 (33-41)	-	-
Female	-	37	-	-
Male	-	36	-	-
Number of patients with liver cirrhosis	7	30	-	15
Female	2	15	-	6
Male	5	15	-	9
Liver fibrosis, kPa	6,0 (4,3-12,0)	24,8 (8,5-36,3)	16,2 (6,8-27,4)	9,0 (6,2-23,3)
Female	5,5	24,8	16,8	11,6
Male	6,8	25,4	15,9	8,8
White blood cells $\times 10^3$ WBC/mm ³	6,5 (5,7-7,0)	5,0 (4,3-6,2)	6,1 (5,1-7,3)	6,0 (5,5-7,7)
Female	6,0	4,9	6,4	6,0
Male	6,8	5,3	6,1	6,4
Haemoglobin, g/dl	14,3 (13,1-15,2)	13,7 (12,3-14,8)	13,7 (12,9-14,9)	14,0 (13,4-15,2)
Female	13,1	13,5	13,6	13,6
Male	14,8	14,1	14,4	14,6
Platelet count, $\times 10^3$ platelets/mm ³	225 (176-259)	109 (75-159)	130 (92-189)	132 (101-203)
Female	244	105	113	124
Male	214	111	137	153
ALT level, IU/mL	29 (19-39)	58 (42-97)	21 (17-31)	20 (15-25)
Female	36	69	23	21
Male	29	57	19	20
AST level, IU/mL	24 (20-29)	60 (37-78)	27 (20-36)	21 (17-28)
Female	27	72	30	22
Male	23	51	24	21
Total bilirubin, μ mol/l	9,3 (6,8-12,2)	14,3 (10,3-21,7)	10,3 (7,4-17,8)	9,0 (7,7-10,7)
Female	7,2	14,3	11,3	9,7
Male	10,3	14,8	9,0	9,0

There were significant changes in the expression of NK receptors and their MFI after the treatment.

First of all, the percentage of NK cells among all lymphocytes was significantly lower in the group of chronically infected comparing with the control group ($p=0,004$). Treatment caused the increase in this percentage in both T1 and T2 ($p=0,01$ and $0,03$, respectively). There were no differences between the control group and the cured population.

We assessed three activating NK receptors: NKG2D, NKp30 and KIR2DS4. Soley expression of KIR2DS4 was elevated on NK cells in patients with CHC ($p=0,009$), and it remained increased after the treatment ($p=0,03$ in T1). Mean Fluorescence Intensity (MFI) of NKp30 was significantly increased in patients with CHC comparing with controls ($p=0,04$), and there was a visible but statistically insignificant increase in MFI of KIR2DS4. After the treatment intensity of fluorescence of those receptors decreased in T1 ($p=0,007$ and $0,0005$, consecutively), while in T2 we observed diminished expression only in KIR2DS4 ($p=0,006$).

We analysed two inhibitory receptors: NKG2A and KIR2DL2/DL3 (CD158b). We did not observe any changes in their expression on NK cells, regardless of HCV status. MFI of NKG2A increased significantly among CHC patients comparing to controls ($p=0,0006$), CD158 was elevated without statistical evidence. After the therapy, MFI of both receptors decreased ($p=0,0004$ and $0,003$ in T1; $p=0,0006$ and $0,03$ in T2, consecutively).

To assess two mechanisms of NK cells cytotoxicity we measured expression and MFI of TRAIL protein and granzyme B. In the study group, we observed increase of granzyme B ($p=0,02$ in T1) and decrease of TRAIL expression on NK cells ($p=0,04$ in T2) after the DAA treatment.

Finally, with ELISA technique, we checked serum levels of NK-chemoattractant chemokine, CXCL10. It was significantly elevated among chronically HCV-infected patients ($p=0,001$), and it decreased after the therapy ($p=0,001$ in T1 and $0,00003$ in T2).

Summary of described changes is presented in Table 3.

	CONTROL	T0	T1	T2
NK/sample %	3,2	2,7	2,8	2,9
NK/lymph %	15,0	<u>9,1</u>	12,7	11,2
NK GRANZ B %	88,6	88,4	92,7	90,1
GRANZ B MFI	7205,0	6872,0	7109,0	6918,0
NK KIR2DS4+ %	1,7	<u>6,8</u>	<u>6,9</u>	3,1
NK KIR2DS4+ MFI	1416	1774,0	1546,0	1306,0
NK KIR2DL2/DL3+ % (cd158b)	28,1	35,8	31,1	29,5
NK KIR2DL2/DL3+ MFI	5142,0	6293,0	4199,0	4668,0
NK NKG2A+ %	42,0	43,7	40,4	38,8
NK NKG2A+ MFI	6454,0	<u>9298,0</u>	7328,5	5364,0
NK NKG2D+ %	76,6	72,9	77,3	85,1
NK NKG2D+ MFI	3033,5	3163,0	2958,0	2669,0
NK TRAIL+ %	0,4	0,7	0,3	0,2
NK TRAIL+ MFI	1172,0	1093,0	999,0	980,0
NK Nkp30+ %	63,9	66,6	65,3	53,9
NK Nkp30+ MFI	2958,0	<u>3198,5</u>	2635,5	<u>2107,5</u>
serum CXCL10 pg/ml	74,8	<u>140,3</u>	91,1	64,9

Table 3 Median values of measured NK cells' parameters in the healthy control group (control), in chronically HCV-infected individuals (T0) and in patients treated with DAA in two consecutive time points (T1 and T2). Underlined numbers differ significantly from those in the control group in Mann-Whitney U test. Highlighted windows present values that changed significantly after the successful treatment in Wilcoxon signed-rank test – blue colour indicates decrease and red means increase.

Afterwards, we compared results described above among genders. Comparing chronically infected patients with the control group, we observed, that MFI of KIR2DL2/DL3 was elevated only in female ($p=0,02$) and MFI of Nkp30 was higher in male ($p=0,016$). There were a few significant differences in NK cells' reaction to HCV eradication between men and women. All the changes are presented in the figures below.

Discussion

After the successful DAA therapy, we observed a decrease in liver fibrosis as well as improvement in biochemical and haematological parameters comparable to the changes presented in the literature³³. Interferon-free HCV therapies are doubtlessly a great achievement of modern medicine.

In the group of patients with CHC, we observed changes in NK cells that partially restored after the virus eradication. Generally, the modifications were consistent with those described in other studies, summed up in table 1. We showed a pattern of changes of activating receptor KIR2DS4 that, with our best knowledge, have not been described in the context of CHC and its' treatment before. This finding enlightens our understanding of the behaviour of NK cells in the course of HCV infection.

Our data showed few differences between genders in NK cells' reaction to the successful treatment of CHC.

NKp30 is a member of Natural Cytotoxicity Receptors family, that is expressed on all mature resting and activated NK cells. This activating receptor plays a central role in resistance to infectious diseases³⁴. High baseline expression of this receptor favours vivid NK cells reaction to infections like e.g. malaria³⁵. It is most likely hormone-dependent since its expression varies in different phases of menstrual cycle³⁶. In various studies, it was either increased or decreased in chronically HCV-infected patients, remaining its behaviour in given disease elusive. There is a theory that in chronically infected patients it persists increased, nonetheless inactive³⁷. Researches showed NKp30 decreases among patients who cleared HCV spontaneously in the acute phase, below the values of healthy population¹⁶. In our study, MFI of NKp30 was increased only in chronically infected male, not in the female. After the treatment, it decreased significantly in women, leaving them with final values lower than in healthy controls. This result may show that female response to infectious diseases may be diminished even after the successful HCV treatment.

Killer cell immunoglobulin-like receptors (KIRs) repertoire of NK cells depends on both KIR and HLA gene polymorphisms³⁸. Depending on KIR genotype, male and female have a different probability of becoming chronically HCV-infected³⁹. Haplotype A of KIRs includes KIR2DL2/DL3 (CD158b), an inhibitory receptor that senses HLA-1 expressing cells and prevents NK cells from auto aggression. Its excessive activation causes susceptibility to malignancies like melanoma and its metastasis³⁸. In children with CHC it is associated with more advanced liver fibrosis⁴⁰. There is a link between KIR2DL2 genes and HCV-related lymphoproliferative disorders⁴¹. Combination of KIR2DL2 presence with HLA-C1 is a significant risk factor of HCC development in HCV-infected patients^{42 43}. Some papers show the receptor's increase in chronic HCV infection, while others prove it remains unchanged. Our data indicate that MFI of CD158b on NK cells increases in HCV-infected female comparing to healthy controls, and normalises after the successful treatment. In the male expression of that receptor remained unchanged in all examined groups, regardless of infection status. In our opinion, its expression in CHC requires further studies.

Activation-induced NK cell death is accompanied by the leakage of granzyme B from intracellular granules into the cytoplasm. This serine protease contributes to target cell death, playing an important role in the development of autoimmunity, e.g., rheumatoid arthritis⁴⁴. T-cells of patients with HCC present diminish the production of granzyme B⁴⁵. Due to its antigen-specific properties, this protease is considered novel therapy for solid tumors⁴⁶. In our study percentage of NK cells expressing granzyme B did not differ between chronically HCV-infected patients and controls but increased in treated men, not women. This finding may suggest male are better protected by NK cells against HCC and autoimmunity after the completed therapy. Since statistically among them recurrence and occurrence rate of HCC is higher²⁸, immunological mechanisms of this phenomenon require further investigations.

C-X-C chemokine 10 (CXCL10) is a chemokine produced by hepatocytes and liver-infiltrating lymphocytes during HCV infection, inducing the migration of T-cells and NK-cells to the liver⁴⁷. High levels of CXCL10 are connected to poor treatment outcomes in patients with chronic hepatitis C and decompensated

cirrhosis treated with DAA⁴⁸. Furthermore, elevated serum concentrations of this chemoattractant are associated with HCV-related mixed cryoglobulinaemia, especially in patients with clinically active vasculitis.⁴⁹ Female gender is one of the main factors related to cryoglobulin production⁵⁰. Interferon-free therapies seem to decrease vasculitis activity in most HCV-patients^{51,52}. In our study, CXCL10 levels declined in women after the therapy, which confirms the significant role of CXCL10 in the pathogenesis of HCV-related mixed-cryoglobulinemia. CXCL10 is speculated to be a candidate for a novel therapeutic target in this condition⁴⁹, and our study supports this concept.

In conclusion, post-menopausal female and male differ in the reaction of their NK cells to interferon-free therapy of CHC. Our research may lead to more studies on different nature of female and male immune systems in the context of HCV infection and treatment.

Declarations

Ethics Statement

This study was carried out following the Declaration of Helsinki of the World Medical Association and was approved by the Ethical Committee of Poznan University of Medical Sciences. All the enrolled study participants met the criteria and completed the study. They were fully informed about the study, and all of them expressed written informed consent before their examination.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

MFI of NKp30 on NK cells before (T0) and after (T1) the treatment in female and male. In Wilcoxon signed-rank test, mean MFI in female decreased significantly ($p=0,01$) while in male did not change.

Figure 1

MFI of NKp30 on NK cells before (T0) and after (T1) the treatment in female and male. In Wilcoxon signed-rank test, mean MFI in female decreased significantly ($p=0,01$) while in male did not change.

Figure 2

MFI of KIR2DL2/3 on NK cells before (T0) and after (T1) the treatment in female and male. In Wilcoxon signed-rank test, mean MFI in female decreased significantly ($p=0,04$) while in male did not change.

Figure 2

MFI of KIR2DL2/3 on NK cells before (T0) and after (T1) the treatment in female and male. In Wilcoxon signed-rank test, mean MFI in female decreased significantly ($p=0,04$) while in male did not change.

Figure 3

Serum levels of CXCL10 before (T0) and after (T1) the treatment in female and male. In Wilcoxon signed-rank test, mean concentration in female decreased significantly ($p=0,002$) while in male did not change.

Figure 3

Serum levels of CXCL10 before (T0) and after (T1) the treatment in female and male. In Wilcoxon signed-rank test, mean concentration in female decreased significantly ($p=0,002$) while in male did not change.

Figure 4

Percentage of NK cells expressing granzyme B in female and male before DAA treatment (T0) and after (T1). In Wilcoxon signed-rank test mean percentage in male increased significantly ($p=0,008$) while in female did not change.

Figure 4

Percentage of NK cells expressing granzyme B in female and male before DAA treatment (T0) and after (T1). In Wilcoxon signed-rank test mean percentage in male increased significantly ($p=0,008$) while in female did not change.