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Analysis of Populations of Memory T-Helper Cells Expressing CXCR3 and CCR6 Chemokine Receptors in Peripheral Blood of Patients with Chronic Viral Hepatitis C

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Flow cytometry was employed to examine the content of major populations of memory T helper cells and expression of chemokine receptors CXCR3 and CCR6 on their surface in peripheral blood drawn from virtually healthy people and the patients with chronic viral hepatitis C. The following combination of monoclonal antibodies had been used: CD62L-FITC/CD45RA-PE/CD3-ECD/CCR6-PC7/CXCR3-APC/CD4-APC-Cy7. In comparison with control group, the patients with chronic hepatitis C had a smaller number of populations of naïve $CD4^+$ T cells and central memory $CD4^+$ T cells but a greater number of terminally differentiated effector memory $CD4^+$ T cells and effector memory $CD4^+$ T cells. No differences were revealed between $CD4^+$ T cell populations of both groups in expression of CXCR3 and CCR6 receptors.

Key Words: *hepatitis C; memory cells; CXCR3; CCR6; CD62L*

Chronic viral hepatitis C (viral CHC) is a grave challenge for modern health care. No less than 3% Earth population is infected with hepatitis C virus (HCV). This disease is characterized with a high incidence of chronization aggravated in many patients with the development of liver cirrhosis, hepatocellular carcinoma, and severe extrahepatic complications. Still there are no adequate vaccination approaches to protect the humans against HCV [4].

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The major role in elimination of HCV is played by T-cell element of the specific immune response, albeit it is insufficiently effective during the primary reaction to infection. Viral CHC inhibits the effector and migration functions of T cells resulting in chronization of the disease and the disturbances in the chemokine system [2,5-7,13].

The chemokines are given the key role in formation of the infection focus. They activate the blood lymphocytes and trigger their migration into various tissues in the organism. The type of prevailing immune response is determined by microenvironment of infection focus. To assess effectiveness of its development during viral CHC, one can examine the changes in the subpopulation composition of T-helper memory cells (T-MC) in peripheral blood of the patients as well as the peculiarities of expression of chemokine receptors (CR) specific of the certain types of immune response.

There are many ways to examine T-MC with flow cytometry. One of the popular approaches is based on the use of CD62L and CD45RA molecules. An adhesion molecule CD62L is responsible for migration of the cells into the secondary lymphoid organs and their T-dependent zones. Pronounced expression of CD62L molecules is observed in naïve T cells ($CD62L^+CD45RA^+$) and central T-MC ($CD62L^+CD45RA^-$). In contrast, effector T cells ($CD62L^-CD45RA^-$) carry no surface CD62L molecules. Alternatively, expression of CD62L molecules is down-regulated during implementation of the basic functions of effector T cells in tissues. Naïve T cells express CD45RA on their surface, and in parallel to maturation and differentiation into MC or effector cells, they replace CD45RA with various transitory forms and CD45R0. However, the terminally differentiated effector T cells with $CD62L^-CD45RA^+$ phenotype also carry out CD45RA on their surface [8]. In the infectious focus, effector T-MC ensure immediate protection against the pathogen, while the central T-MC located in the secondary lymphoid organs produce novel generations of effector T cells. In population of T-MC, terminally differentiated effector T cells are most differentiated. More than 60% cells of this population do not express the costimulatory molecules CD27 and CD28 [3]. The cells in T-MC population implement especially important effector functions. The effector Th1-MC and Th2-MC are predominantly belongs to population of effector T-helper cells, while Th1 and Th2 effector cells are situated in the population of terminally differentiated effector T helpers.

Most works on T-MC focus predominantly on cytotoxic T lymphocytes. In particular, under conditions of various viral infections, the specific $CD8^+$ T cells can be located in different populations of cytotoxic T-MC [10]. In contrast, few studies were focused on the populations of T-MC under the viral infections.

However, numerous investigations showed that various populations of T helpers express a certain repertoire of surface CR. For instance, Th1 lymphocytes express CXCR3 and CCR5 receptors, while Th2 and Th17 express CCR4 and CCR6 receptors, correspondingly [9].

Based on the expression of CXCR3 and CCR6 receptors, the researchers single out $CXCR3^+CCR6^-$, $CXCR3^+CCR6^+$, and $CXCR3^-CCR6^+$ populations of $CD4^+$ lymphocytes, which express the cytokines and differentiation factors that are specific of various differentiated lines of T helpers: Th1, Th1/Th17, and Th17 [12].

Similarly, expression of CXCR3 or CCR4 receptors in central $CD4^+$ T-MC identifies the pre-Th1 and pre-Th2 lymphocytes [14]. In comparison with effector $CD4^+$ T-MC exposing the above CR, these lymphocytes have a smaller ability to produce IFN-γ or IL-4, so they differentiate into Th1 and Th2 in response to homeostatic cytokines IL-7 and IL-15 correspondingly, irrespective of the standard stimulators that polarize T helpers.

Thus, the study of expression of CD45RA and CD62L (the differentiation markers of T cells) as well as expression of activation and migration markers CXCR3 and CCR6 in population of T helpers can expand our views on formation of the immune memory, the development of immune response, and activation and homing of T-helper lymphocyte populations during viral CHC.

Our aim was to assay $CD4^+$ T-MC and to quantify expression of CXCR3 and CCR6 CR on their surface in the peripheral blood of patients with viral CHC.

MATERIALS AND METHODS

The study comprised 29 persons with established viral CHC, who did not receive the standard therapy with IFN and Ribavirin. In addition, they were not infected with HIV or hepatitis B virus. The outpatient control was performed in S. P. Botkin Municipal Hospital for Infectious Diseases No. 30 (St. Petersburg). The diagnosis was established after documenting the presence of anti-CHC antibodies and revealing the viral RNA with PCR. The control group (n=27) comprised age- and sex-matched virtually healthy donors without somatic diseases and clinical diagnostic and morphological signs of hepatic lesion. All examinees were the residents of St. Petersburg, Leningrad Region, or North-Western Area in Russian.

Immunophenotyping of T-helper lymphocytes was performed by six-color flow cytofluorometry using a set of monoclonal antibodies conjugated with fluorochromes: CD62L-FITC/CD45RA-PE/CD3-ECD/CCR6-PC7/CXCR3-APC/CD4-APC-Cy7, CD62L-FITC, CD45RA-PE, CD3-ECD (Beckman Coulter) and CCR6-PC7, CXCR3-APC, CD4-APC-Cy7 (BioLegend). Venous blood was stained with monoclonal antibodies [1]. The erythrocytes were lysed with no-wash-procedure using VersaLyse Lysing Solution (Beckman Coulter). The samples were analyzed in a Navios Flow Cytometer (Beckman Coulter). In each sample, not less than 50,000 lymphocytes gated by the parameters of a small-angle or side light scattering were examined. The absolute number of cells was obtained in a single-platform system employing a Flow-Count reagent (Beckman Coulter)

The data were analyzed statistically using Navios Software 1.2 (Beckman Coulter), Kaluza 1.2 (Beckman Coulter), GraphPad Prizm 6, and GraphPad software with non-parametric Mann–Whitney test at $p<0.05$.

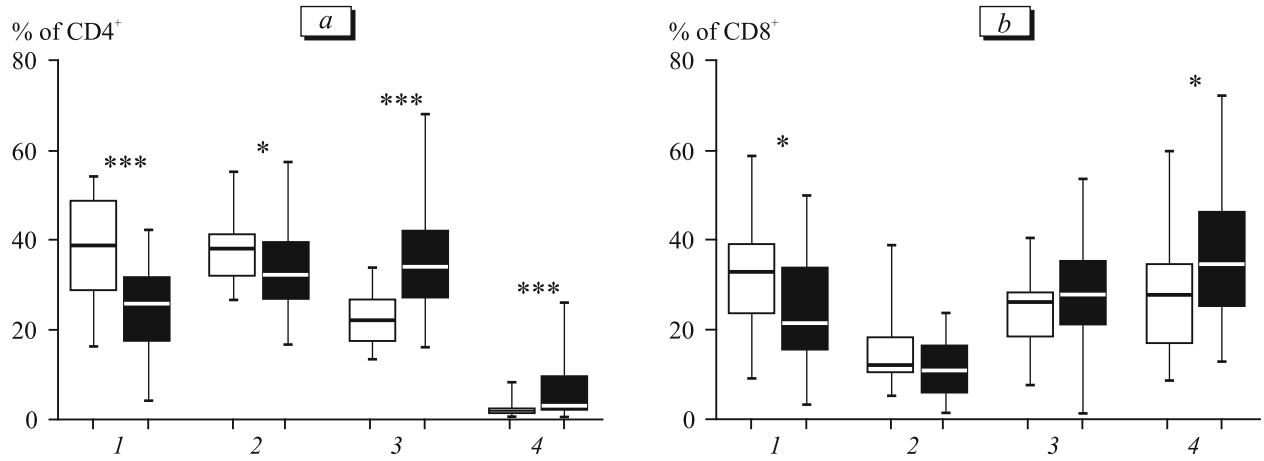

Fig. 1. Subpopulation composition of T helper (*a*) and T cytotoxic (*b*) lymphocytes. Here and in Fig. 2: *1*) naïve; *2*) central; *3*) effector; and *4*) terminally differentiated effector (TEMRA) subpopulations. The light and dark bars correspond to healthy (control) persons and the patients with viral CHC. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ in comparison with the control.

TABLE 1. Content of T Helper Cells Expressing CXCR3 and CCR6 in Peripheral Blood of Patients with Viral CHC and in Virtually Healthy Persons (Me (Q25; Q75))

Population of CD4+ T cells		Control (*N*=27)	Viral CHC (*N*=29)	*p*
Naïve	%	38.76 (28.56; 48.73)	25.74 (17.65; 31.54)	<0.0001
	10^6/liter	292.1 (201.4; 380.7)	220.7 (145.6; 302.7)	0.033
CXCR3+ naïve	%	1.55 (1.10; 2.96)	1.04 (0.76; 1.26)	0.0017
	10^6/liter	14.67 (10.55; 17.19)	8.37 (6.22; 15.10)	0.0199
CCR6+ naïve	%	0.92 (0.50; 1.38)	0.34 (0.22; 0.65)	0.0007
	10^6/liter	6.11 (3.63; 13.09)	3.17 (1.91; 5.31)	0.0011
Central	%	38.43 (31.73; 41.14)	32.38 (26.80; 39.34)	0.0257
	10^6/liter	295.1 (231.7; 385.5)	292.2 (203.3; 407.3)	0.8312
CXCR3+ central	%	16.20 (14.55; 19.33)	14.34 (10.89; 18.94)	0.2067
	10^6/liter	117.0 (102.1; 181.6)	125.7 (90.2; 200.6)	0.7554
CCR6+ central	%	15.20 (12.10; 18.51)	11.42 (8.76; 14.67)	0.0016
	10^6/liter	108.0 (94.9; 146.8)	108.1 (84.0; 127.6)	0.149
Effector	%	22.10 (17.58; 26.81)	34.08 (26.95; 41.83)	<0.0001
	10^6/liter	158.2 (125.7; 238.2)	298.4 (227.3; 420.2)	<0.0001
CXCR3+-effector	%	15.10 (11.33; 18.39)	24.10 (19.62; 29.43)	<0.0001
	10^6/liter	113.6 (82.4; 161.9)	189.1 (163.6; 309.6)	<0.0001
CCR6+-effector	%	13.70 (9.77; 16.52)	17.88 (12.42; 22.44)	0.011
	10^6/liter	94.1 (73.7; 148.9)	155.0 (121.7; 194.4)	0.0043
Terminal-differentiated excitatory (TEMRA)	%	1.52 (0.91; 2.36)	3.39 (1.89; 9.55)	0.0004
	10^6/liter	12.39 (7.03; 20.44)	30.12 (18.15; 79.69)	0.0002
CXCR3+-TEMRA	%	0.79 (0.53; 1.37)	1.01 (0.81; 2.40)	0.0163
	10^6/liter	5.20 (3.80; 10.58)	11.61 (6.33; 24.05)	0.0035
CCR6+-TEMRA	%	0.35 (0.23; 0.42)	0.44 (0.15; 0.77)	0.2611
	10^6/liter	2.59 (2.04; 3.57)	4.34 (1.57; 5.91)	0.2009

RESULTS

The data on expression of CD62L and CD45RA molecules were used to determine the levels of naive $CD4^+$ T cells and the following populations of $CD4^+$ T-MC: central, effector, and terminally differentiated effector T-MC (Fig. 1).

In patients with viral CHC, the levels of effector and terminally differentiated effector T-MC was enhanced in comparison with control, while the content of naïve T helpers and central $CD4^+$ T-MC was diminished (Fig. 1, *a*).

In these patients, the populations of T-MC are redistributed predominantly due to naïve MC in favor of the effector cells that are responsible for immediate defense against viral aggression in the affected tissue. The paper [15] reports similar observations except for enhanced level of central T-MC, which can be explained by the use of CD27 molecule in counting T-MC instead of CD62L molecule employed in our study.

It is not a simple routine to reveal specific T helpers in patients with viral CHC, because they mostly appear during the acute phase of the disease or in convalescents with $CCR7^+CD45RA^-CD27^+$ phenotype [11] characteristic of central memory cells, whose level was diminished in our study. This specificity can be also explained by the use of CD62L marker in this work, which differs in expression from CD27 and CCR7 molecules.

We next examined the population of $CD4^+$ T lymphocytes at various differentiation stages and determined *1*) the level of the cells expressing CR CXCR3 and CCR6, *2*) the shares of $CXCR3^+$ and $CCR6^+$ cells among T helpers, and *3*) the level of these cells.

The populations of $CD4^+$ T-MC in the compared groups differed by the expression of CXCR3 and CCR6. In both groups, naïve $CD4^+$ T-MC virtually did not express the examined CR (Fig. 2). It agrees with published data that this population of T cells has no receptors for pro-inflammatory chemokines

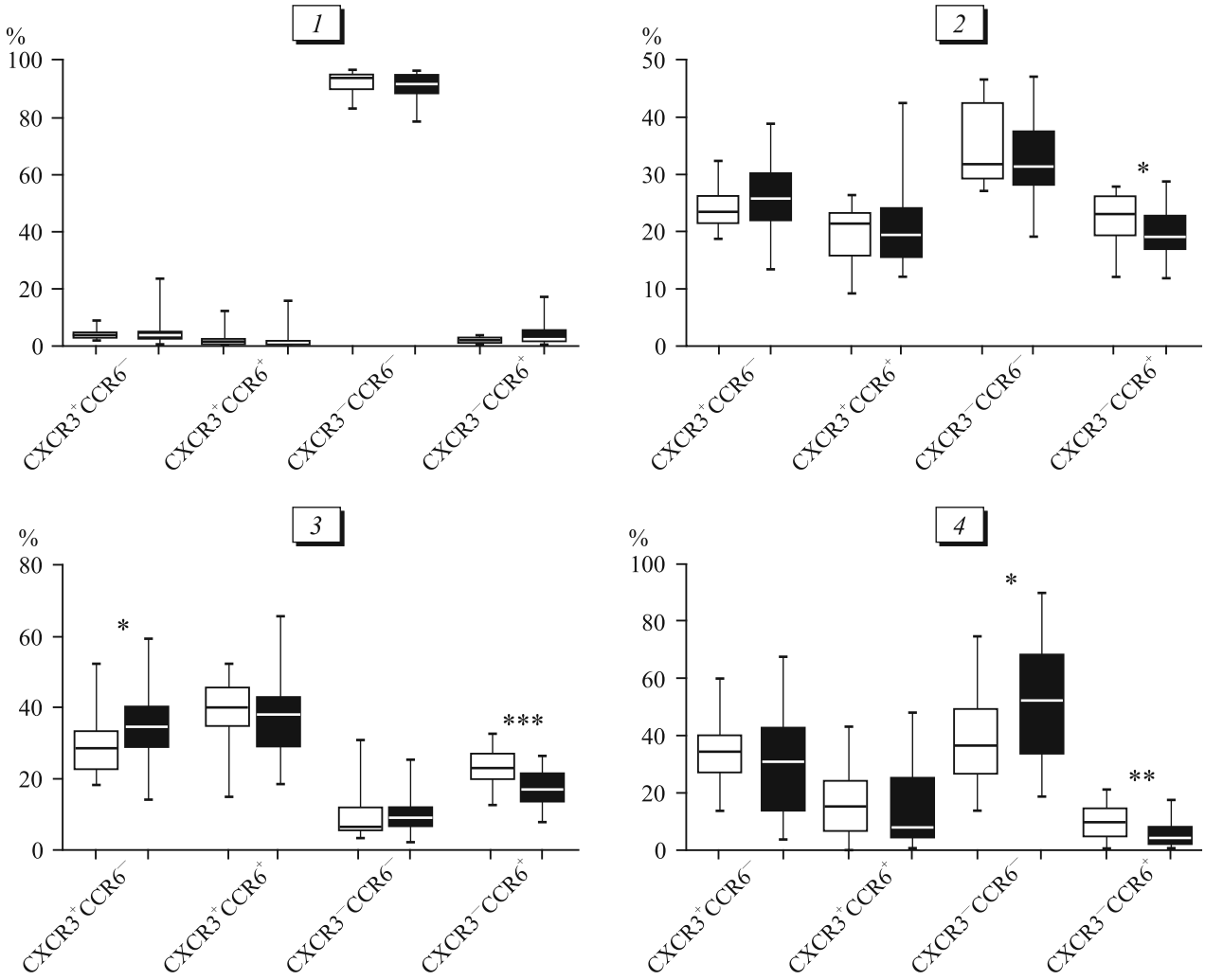

Fig. 2. Subpopulation expressing CXCR3 and CCR6 in populations of T-MC.

CCR1, CCR3, CCR5, and CXCR3 and adhesion molecules CD11b, CD29, and CD49e whose ligands are expressed on activated endotheliocytes of the microcirculatory bed.

In contrast, during viral CHC, the number of cells with $CXCR3^{-}CCR6^{+}$ phenotype decreased in all populations of $CD4^{+}$ T-MC, which can attest to inhibition of Th17 immune response associated with this phenotype during the disease. The $CD4^{+}$ T-MC population of effector cell was characterized with an enhanced number of subpopulation $CXCR3^{+}CCR6^{-}$ cells, which probably relates to re-distribution of the cells in favor of Th1 response observed in most patients with this disease. No changes in the level of $CXCR3^{+}CCR6^{-}$ cells were revealed in population of central T-MC with pre-Th1-like phenotype [14]. In contrast, the population of terminally differentiated effector $CD4^{+}$ T-MC demonstrated an increased number of cells with $CXCR3^{-}CCR6^{-}$ phenotype (Fig. 2), which probably results from the loss of CXCR3 and CCR6 receptors by some of these cells during formation of the immune response.

In comparison with control group, the patients with viral CHC were characterized with enhanced levels of $CXCR3^{+}$ effector, $CXCR3^{+}$ terminally differentiated effector, and $CCR6^{+}$ effector T helpers, while they had decreased levels of $CXCR3^{+}$ naïve cells, $CCR6^{+}$ naïve cells, and $CCR6^{+}$ central T helpers (Table 1). However, the level of subpopulation of $CCR6^{+}$ cells in central $CD4^{+}$ T-MC decreased only relatively. This study demonstrated an elevated number of $CXCR3^{+}$ and $CCR6^{+}$ effector MC resulting from up-regulation of expression of these receptors during viral CHC, which can attest to enhanced (in comparison with other populations) migration of T helpers with $CD62L^{-}CD45RA^{-}$ phenotype in hepatic tissue during viral CHC.

Thus, the peripheral blood of patients with viral CHC is characterized with *1*) re-distribution of $CD4^{+}$ T cells in favor of their effector populations; *2*) an elevated contents of effector and terminally differentiated effector T helpers expressing CXCR3; and *3*) an enhanced level of effector T helpers expressing CCR6. In addition, this blood demonstrates a different expression of CXCR3 and CCR6 receptors in T-MC with increased content of $CXCR3^{+}CCR6^{-}$ cells in population of effector T-MC as well as a decreased content of $CXCR3^{-}CCR6^{+}$ cell subpopulation among all $CD4^{+}$ T-MC populations.

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