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Cytokine-Mediated Regulation of Expression of *Gfi1* and *U2afll4* Genes by Activated T-Cells with Various Differentiation Status in vitro

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Abstract—The dose-dependent effects of cytokines (IL-2, IL-7, and IL-15), which have a common γ-chain, on mRNA expression of *U2afll4* and *GFi1* genes involved in regulation of alternative splicing of the *Ptprc* gene, have been investigated in vivo using T-lymphocyte cultures with different degrees of differentiation. IL-2, IL-7, and IL-15 caused a similar unidirectional inhibitory effect of various severity on restimulated CD45RO$^+$ T-cells exposed to an antigen-independent activation; they caused a dose-dependent decrease of the *U2af1l4* gene expression, and an increase of *Gfi1* gene expression. This may suggest formation of active forms of the CD45 receptor, and also limitation of the formation of low-molecular short splice variants of the CD45RO receptor. Under conditions of antigen-independent stimulation of naive CD45RA$^+$ - cells rIL-7 and IL-15 exhibited opposite effects on *U2af1l4* and *Gfi1* gene expression. The increase of IL-7 concentrations in the incubation medium of naive cells was accompanied by a decrease in expression of both genes. IL-15 IL-7 exhibited opposite effects. Cytokines possessing a common γ-chain (IL-2, IL-7, and IL-15) prevented antigen-independent differentiation of naive T-cells, by preventing the formation of polyclonal "surrogate" cells. In general, the study of the molecular mechanisms of genetic control determining homeostatic processes of T-cells in response to exposure to antigenic or non-antigenic treatments may be important in the construction of a general model of self-maintenance and differentiation of immune cells.

Keywords: cytokines, gene expression, T-cells, activation, alternative splicing

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INTRODUCTION

The cycle of functional changes that occur in response to antigenic stimulation of T-cells requires corresponding changes in the expression of numerous proteins. Alternative splicing is the main mechanism for regulation of gene expression, by which it is possible to obtain several unique mRNAs of a single gene by selective inclusion or exclusion of exons [1]. Recent studies demonstrate that primary transcripts of almost 95% of all human genes are alternatively spliced; among these genes, the genes expressed in cells of the nervous and immune systems dominate [2, 3]. The importance of regulation of alternative splicing in the humans may be illustrated by the *Ptprc* gene; its pre-mRNA consists of 33 exons and encodes a common leukocyte receptor CD45 [4, 5]. The CD45 molecule is a transmembrane tyrosine protein phosphatase, which has been recognized as a critical regulator of signaling mediated by a T-cell receptor (TCR) [6, 7]. Naturally occurring polymorphisms in the CD45 gene, correlate with susceptibility to a wide range of autoimmune diseases, viral infections and severe human immunodeficiency, particularly to multiple sclerosis, and HIV infection [8]. One of the mechanisms regulating alternative splicing of the *Ptprc* gene is based on the opposite effects of the splicing factor U2af1l4 (U2 small nuclear RNA auxiliary factor 1-like 4, U2af1l4) and the transcription factor Gfi1 (growth factor independent 1). This, finally, determines formation of various CD45 isoforms and activation of T-cells during the immune response [9, 10]. Various CD45 isoforms exhibit different effects on the function of T-cells due to differences in their ability to interact with the ligand, between themselves or with TCR [11].

Cytokines of type I family (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21), which share a common γ-chain, can exert complex effects on homeostasis of T-cell lymphocyte of different degree of differentiation [12–14]. In the present study we have investigated the influence of cytokines, with a common γ-chain (IL-2, IL-7, and IL-15) on changes in mRNA levels of *Gfi1* and *U2af1l4* genes, determining the ratio of the major variants of the CD45 receptor in cell cultures, with different degrees of differentiation: naive T-lymphocytes

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(CD45RA$^+$) and memory T-cells (CD45RO$^+$) obtained from healthy donors.

MATERIALS AND METHODS

Venous blood of 20 healthy donors (9 men and 11 women) aged from 19 to 39 years was used in expeirments. Blood is taken using standard vacuum systems BD VACUTAINER^{TM} (Greiner-bio-one, Austria) with heparin (20 U/mL).

Populations of naive and primed lymphocytes were obtained from isolated mononuclear cells (MNC) by immunomagnetic separation using paramagnetic particles with monoclonal antibody to the CD45RO$^+$ and CD45RA$^+$ (MACS CD45RO Microbeads, human, Miltinyi Biotec, Germany) and a AutoMACS Pro Separator Instrument (Miltinyi Biotec) according to the manufacturer's protocol.

Purity of the isolated cells (CD45RA$^+$; CD45RO$^+$) was determined by means of monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Abcam, UK). The percentage of positive cells was determined by a MACSQuantAnalyzer flow cytometer (MiltenyiBiotec). The content of the target fraction of CD45RA$^+$ and CD45RO$^+$ cells in the analyzed samples was not less than 98%.

The absence of monocyte (CD14$^+$) and B-lymphocyte (CD19$^+$) contaminations in the cultures of CD45RA$^+$ and CD45RO$^+$ cells before and after cultivation was confirmed by flow cytometry using monoclonal antibodies conjugated with FITC, PE, PE-Cy7, and PerCP (Abcam, UK and e-Bioscience, USA). Analysis of surface markers was carried using a flow cytometer MACS Quant (Miltenyi Biotec) in accordance with manufacturers' protocols. The cell cultures in which the content of CD3$^+$ CD45RA$^+$ CD14-CD19- and CD3$^+$ CD45RO$^+$ CD14-CD19- cells was 98.1 $\pm$ 1.12% were used in experiments.

The number of live and dead cells in the cultures of CD45RO$^+$ and CD45RA$^+$ lymphocytes as well as the dynamics of changes in the number of cells per mL before and after cultivation was determined by flow laser dual-color cytometry on a flow cytometer Guava EasyCyte Plus using the reagent and program Guava ViaCount (Millipore, USA).

Statistical processing of the results was performed using the statistical software package SPSS_20. For quantitative parameters mean and standard deviation (M $\pm$ SD) were calculated. Differences in parameters between two groups were evaluated by the Wilcoxon test for paired samples.

CD45RA$^+$ and CD45RO$^+$ cells (1×10^6 cells/mL) were cultivated in 48-well plates in serum free Iscove's medium (Sigma, USA) containing 0.5% human serum albumin (Microgen, Russia), 5×10^{-5} M β-mercaptoethanol (Acros Organics, USA) and 30 μg/mL gentamycin in the presence of various concentrations of recombinant forms of cytokines IL-2, IL-7, IL-15 (Miltenyi Biotec) or without cytokines (control) for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. The T-Cell Activation/Expansion Kit human (Ac/Exp) (Miltenyi Biotec) was used as the activator of T-lymphocytes; it represents anti-biotin MACSiBead^{TM} particles with biotinylated antibodies against human CD2$^+$, CD3$^+$, CD28$^+$. The amount of added Ac/Exp reagent (5 μL) contained 0.5×10^6 anti-biotin MACSiBead^{TM} particles. The ratio of cells and activating particles was 1 : 2.

The following variants of cultivation have been used: (1) intact sample; (2) sample with addition of Ac/Exp; (3) samples with addition of Ac/Exp and various concentrations of rIL-2 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); (4) samples with addition of Ac/Exp and various concentrations of rIL-7 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); (5) samples with addition of Ac/Exp and various concentrations of rIL-15 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL).

After incubation, the cell culture total RNA was isolated using an aqueous solution of phenol and guanidine isothiocyanate (ExtractRNA kit Evrogen, Russia), according to the manufacturer's protocol. The concentration of the resulting RNA was measured using a NanoVue Plus spectrophotometer (GE Healthcare, USA). The purification degree of RNA preparations was determined by the ratio of A_{260}/A_{280}. RNA concentrations of the samples were normalized to obtain the same amount of cDNA by adding water to adjust RNA concentration to 10 μg/mL. The resultant samples were used for reverse transcription, which was performed using a MMLV RT reagent kit (Evrogen). cDNA was synthesized according to the manufacturer's protocol and using an oligonucleotide primer (oligo(dT23), 20 μM).

The relative levels of gene expression were evaluated by multiplex PCR using specific TaqMan probes (Evrogen). PCR was performed using specific reagents qPCRmixHS (Evrogen) and the primers (at a concentration of 10 pM), 3 μL of cDNA as a template and the GAPDH gene as a reference gene.

Oligonucleotide primers were synthesized by the phosphoramidite method using an AMS2000 DNA/RNA synthesizer (Biosset, Russia) and reagents from Glen Research (USA). Primers were purified by reverse phase chromatography on a device for oligonucleotide purification (OPS1000 model, Biosset). The following oligonucleotide primers were used:

GFI1_for 5'-TGGAGCAGCACAAAGCC-3'

GFI1_rev 5'-GACAGTGTGGATGACCTCTTG-3'

GFI1_probe FAM-5'-CGCAGGAACGGAG-CTTTGACTGTA-3'~BHQ-1 (probe)

U2af1l4_for 5'-CTTCACAACAAGCCGA-CATTC-3'

U2af1l4_rev 5'-CAAGGTTGTCGCACACATTC-3'

U2af1l4_probe FAM-5'-CCAGGAGGTGTTCACAGAACTGCA-3'~BHQ-1 (probe)

GAPDH_for 5'-GAAGGTGAAGGTCGGAGTC-3'

GAPDH_rev 5'-GAAGATGGTGATGGGATTTC-3'

GAPDH_probe HEX-5'-CAAGCTTCCCGTTCTCAGCC-3'-BHQ-1 (probe).

Specificity of the primers, amplicons, and probes was initially assessed using the on-line program BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

PCR reaction was performed in triplicates using a LightCycler 480 Real-Time PCR thermocycler (Roche, Switzerland) in the following regime: 95°C, 5 min; 95°C, 20 s; 60°C, 30 s; 72°C, 60–55 cycles, 72°C, 5 min. The melting temperature of the amplification product was determined individually for each primer pair during analysis of the melting curve.

Relative expression levels of the investigated genes were calculated using a modified Pfaffl formula for different amplification efficiency. In our case the efficiency of the reaction (E) was equal to 2.

$$\text{Relative expression level} = \frac{E_{\text{exper}}\Delta CP_{\text{exp(contr-exper)}}}{E_{\text{ref}}\Delta CP_{\text{exp(contr-exper)}}}.$$

Calculation of the relative expression level of the investigated gene is based on its effectiveness in real-time PCR (E) and the difference (Δ) of the cross-points (CP) of the unknown (experimental, exper) sample compared with the control ($\Delta CP = CP_{\text{control}} - CP_{\text{exper}}$).

Statistical treatment of the results was performed using the statistical software package SPSS_20. Comparative analysis was performed using the nonparametric Wilcoxon test for paired samples. Differences were considered as statistically significant at $p < 0.05$.

All the healthy donors (volunteers) participated in the study signed informed consent. The authors declare no conflict of interest related to the manuscript.

RESULTS AND DISCUSSION

The total number of cells in intact cultures of CD45RA$^+$ and CD45RO$^+$ T-lymphocytes cultivated for 48 h was $(1.08 \pm 0.09) \times 10^6$ cells/mL. Incubation of T-cells with the Ac/Exp activator, simulating the action of antigen presenting cells (APC), was accompanied by an increase in the cell number in both cultures (CD45RA$^+$ and CD45RO$^+$) T-lymphocytes by about 22% (Fig. 1). Analysis of lymphocyte viability has shown that incubation of the cultures of CD45RA$^+$ and CD45RO$^+$ T-cells with the Ac/Exp activator resulted in a statistically significant reduction in the number of viable lymphocytes compared with the intact control (incubated without this activator). The activator caused a more pronounced decrease in the viability of CD45RA$^+$ T-cells (by 25%) (Fig. 2). Such a reduction can be due to activation of apoptosis, developed along with the increased proliferative response [10, 15, 16].

The maximum concentration of rIL-2 had a different opposite effects on the viability of Ac/Exp activated CD45RA$^+$ and CD45RO$^+$ T-cells: it caused a 20% decrease in the number of CD45RO$^+$ T-cells and a 1.5-fold increase in the number of viable CD45RA$^+$ T-cells as compared with the sample containing only the activator as the additive.

Interaction of IL-2 with a high affinity receptor on T-lymphocytes after antigenic stimulation triggers signal events directly regulating the entry of resting T-lymphocytes in the cell cycle [17]. Controversial information exists on the role of IL-2 in clonal expansion of primed CD4$^+$ and CD8$^+$ T-lymphocytes in vivo [11, 13]. Usually, authors indicate involvement of IL-2 in this process by inducing apoptosis of primed CD4$^+$ and CD8$^+$ cells [13]. Realization of the apoptosis-inducing effect of rIL-2 on blood lymphocytes in vitro requires a specific threshold concentration of this cytokine. IL-2 can enhance the expression of such proapoptotic proteins as TRAIL, caspase-3, DAP (death-associated protein) and STK 17B (serine/threonine kinase 17B) [18].

Addition of the maximal concentration (1×10^{-9} g/mL) of rIL-7 or rIL-15 to the culture of activated T-cells of different degree of differentiation, increased the number of viable CD45RO$^+$ T-lymphocytes (by 20% as compared with the cell samples treated only with the activator). These results are consistent with the biological role of IL-7 and IL-15 in T-cell homeostasis and are partially supported by literature data. Survival and homeostatic proliferation of memory T cells in vivo after elimination of the pathogen are determined by the presence cytokines IL-2, IL-7, and IL-15 in their microenvironment [19], and do not depend on interaction of their receptor structures with products of the major histocompatibility complex (MHC) class I (for CD8$^+$) and MHC class II (for CD4$^+$) [20–22]. Naive resting T-cells receive low level signals through their contact with IL-7 and MHC molecules, which help cells to survive for long periods in the resting state without undergoing antigen-independent differentiation [13, 15, 20, 23].

Alternative splicing is one of the mechanisms regulating gene activity in the cells of innate and adaptive immunity [24]. As mentioned above, the antagonistic interactions of U2AF1l4 and Gfi1 factors, regulating

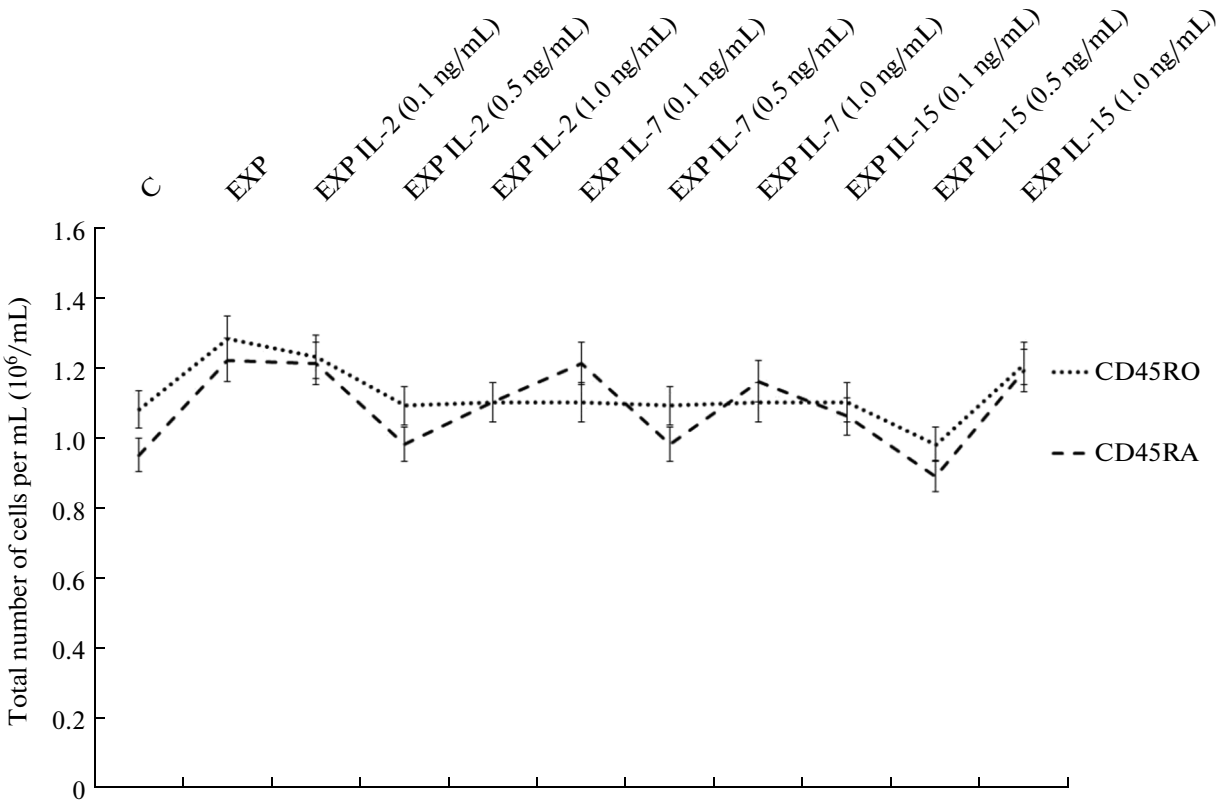

Fig. 1. The total number of cells (per mL) in cultures of $CD45RA^+$ and $CD45RO^+$ lymphocytes during their in vitro cultivation with added activator and various concentrations of cytokines (IL-2, IL-7, IL-15). C is the number of cells per mL in a control sample; Exp – with the T-cell activator; Exp IL2-0.1-0.5-1.0—incubation of cell cultures with T-cell activator and rIL-2 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); Exp IL7-0.1-0.5-1.0—incubation of cell cultures with the T-cell activator and rIL-7 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); Exp IL15-0.1-0.5-1.0—incubation of cell cultures with the T-cell activator and rIL-15 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL).

alternative splicing of transmembrane tyrosine phosphatase CD45, finally determine the formation of CD45 different isoforms and the antigen-dependent T-cell activation [9, 10]. Expression of the CD45 receptor isoforms in immunocompetent cells is strictly controlled during their development, differentiation and activation. Naive T-lymphocytes express predominantly long, high molecular weight isoform CD45 (CD45RA), whereas activated (primed) memory T cells express the short splice variant CD45 (CD45RO), with which more rapid and effective antigen mediated activation is associated [9, 10, 25].

Addition of the T-cell activator in the cultures of $CD45RA^+$ and $CD45RO^+$ T-cells was accompanied by a sharp inhibition of *U2af1l4* expression, especially in the populations of $CD45RO^+$ cells (more than 50-fold) (Fig. 3, 4). The level of *Gfi1* gene transcription in primed T-cells ($CD45RO^+$) was comparable to the control, while in naive T-cells, it decreased by more than 16-fold. In our opinion, these changes (inhibition of *U2af1l4* gene transcription) in cultures of activated T-cells with different degrees of differentiation after incubation for 48 h, associated with the activation of proliferation, are typical for formation of the transition active forms of the CD45 receptor. Heyd et al. [9] showed that the inhibition of *U2af1l4* gene transcription contributes to the formation of active forms of the CD45 molecules, whereas a decrease in the *Gfi1* levels leads to the formation of a shorter version of the *Ptprc* gene transcript.

At the same time, suppression of *Gfi1* gene expression, along with suppression of *U2af1l4* gene expression (in the case of $CD45RA^+$ T-lymphocytes) may be attributed both to the low sensitivity of naive T-cells to antigen-independent stimuli, and to duration of T-cell cultivation. After 24–72 h of T-cell stimulation there was a significant activation of the splicing factor U2AF26 gene, whereas maximal induction of protein Gfi1 synthesis (resulted in appearance of various isoforms) was observed 6–12 h later but was completely absent on day 3 [9]. Our earlier results obtained on a 48 h-culture of mononuclear leukocytes (MNC) dem-

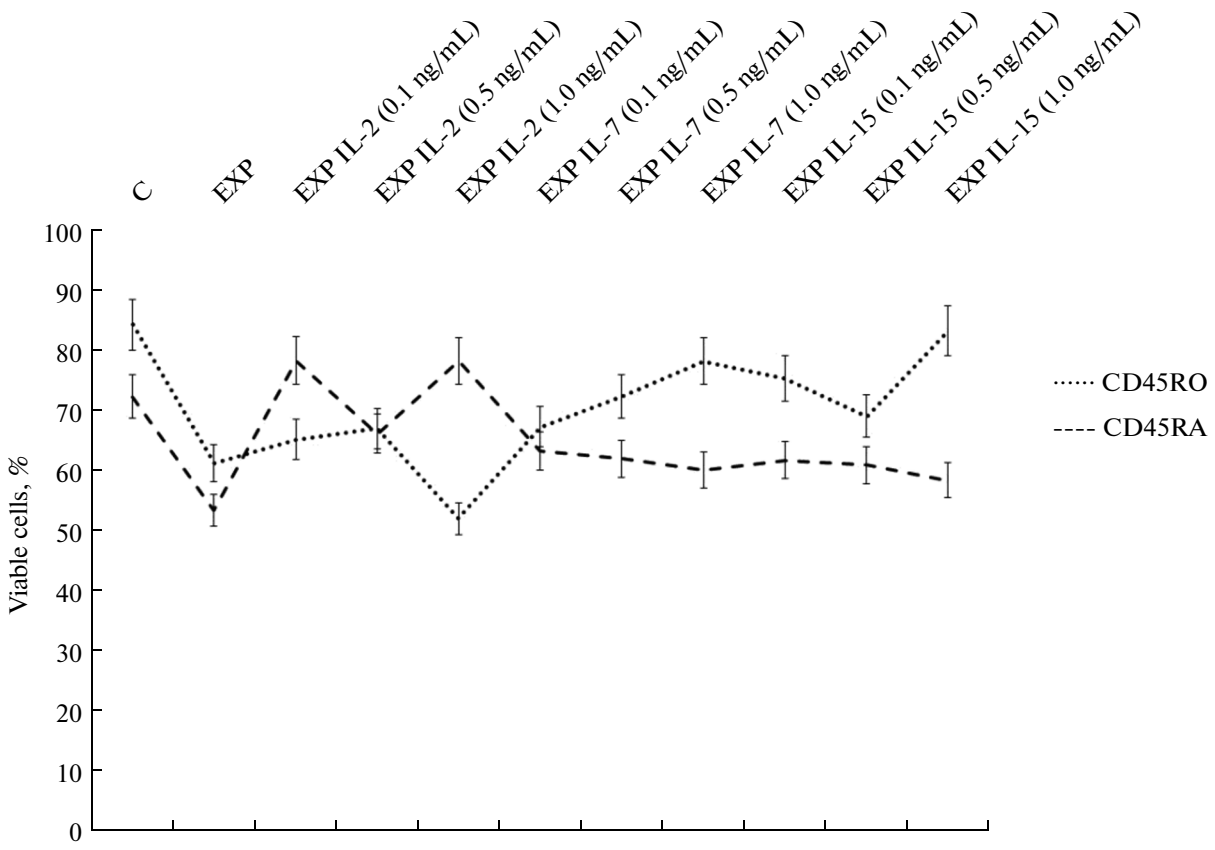

Fig. 2. The content of living cells (%) in cultures of $CD45RA^+$, and $CD45RO^+$ lymphocytes cultivated in vitro with added activator various concentrations of cytokines (IL-2, IL-7, IL-15). C is the number of cells per mL in a control sample; Exp—incubation with the T-cell activator; Exp IL2-0.1-0.5-1.0—incubation of cell cultures with T-cell activator and rIL-2 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); Exp IL7-0.1-0.5-1.0—incubation of cell cultures with the T-cell activator and rIL-7 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); Exp IL15-0.1-0.5-1.0—incubation of cell cultures with the T-cell activator and rIL-15 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL).

onstrate transcriptional activation of U2af1l4, and suppression of its antagonist, Gfi1 [10].

Interesting results were obtained during incubation of cells with different degrees of differentiation with combinations of the activator (Exp) and the recombinant forms of cytokines (IL-2, IL-7, and IL-15). Addition of Exp/rIL-2 combinations to the culture of naive $CD45RA^+$ lymphocytes increased the number of cells and their viability, and also caused a dose-dependent, but changes in mRNA expression of both genes, *U2af1l4* and *Gfi1*. Increased concentrations of rIL-2 decreased transcription of the *U2af1l4* gene, but increased transcription of the gene *Gfi1*; this suggests (although indirectly) formation of active intermediate splice variants of the CD45 receptor (Fig. 3).

IL-7 also exhibited the inhibitory effect on activated naive cells: it caused a simultaneous decrease in mRNA expression of *U2af1l4* and *Gfi1* genes. The most potent inhibition of U2af1l4 and Gfi1 gene expression was observed at 0.5×10^{-9} g/mL rIL-7. The action of IL-15 was opposite to that of IL-2 and IL-7: increasing concentration of IL-15 caused an increase in expression of both genes; however this increase did not reach the values observed in activated control samples (with the addition of the activator). A significant inhibition of *Gfi1* gene expression in activated naive T-cells induced by the cytokines with common γ-chain, can be attributed to the effects of the cytokines, influencing maintenance of cell viability and homeostatic proliferation, rather than activation of naive T-cells, and also to the duration of cell culture incubation (48 h) [10]. In general, cytokines, sharing a common γ-chain (IL-2, IL-7, and IL-15), prevent antigen-independent differentiation of naive T-cells, by preventing the formation of "surrogate" cells.

Dose-mediated effects of IL-2 on restimulated $CD45RO^+$ T-cells were associated with their increased death and were accompanied by inhibition of *U2af1l4* expression with a simultaneous increase in transcription of the *Gfi1* gene, responsible for the for-

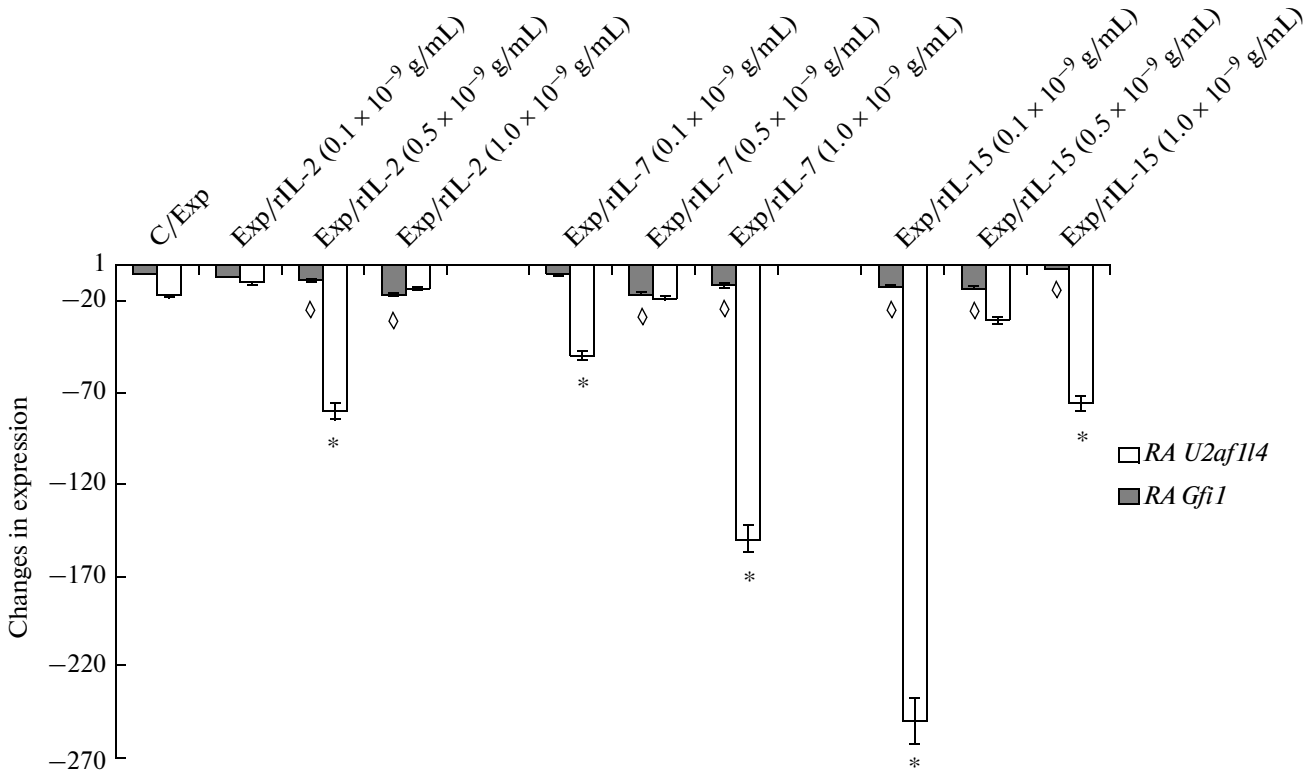

Fig. 3. Relative changes in the level of transcription of *U2af1l4* and *Gfi1* genes and in CD45RA$^+$ T-cells induced by the activator and cytokines (IL-2, IL-7, IL-15) (fold change); C/Exp—the ratio of mRNA expression levels in control (untreated) samples and the samples incubated with additions of the T-cell activator (Exp); Exp IL2-0.1-0.5-1.0—the ratio of mRNA expression levels in samples incubated with additions of the T-cell activator and in samples with additions of rIL-2 0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); Exp IL7-0.1-0.5-1.0—the ratio of mRNA expression levels in samples incubated with additions of the T-cell activator and in samples with additions of rIL-7 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); Exp IL15-0.1-0.5-1.0—the ratio of mRNA expression levels in samples incubated with additions of the T-cell activator and in samples with additions of rIL-15 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL). Here and in Fig. 4: *—significance of differences in *U2af1l4* gene expression as compared with the C/Exp sample; ◇— significance of differences in *Gfi1* gene expression compared with the sample C/Exp. The reduction in the expression levels (fold change) of studied genes was demonstrated using negative values. For example, if the relative levels of gene expression *U2af1l4* is 0.02 (less than 1), this means that the transcription of this gene is 50-times lower as compared with the intact cells.

mation of active intermediate splice variants of the CD45 receptor. IL-7 and IL-15 had a similar effect on the activated CD45RO$^+$ cells: the maximal expression of the *U2af1l4* gene was observed after addition of minimal concentrations of these cytokines, the time-course of changes in *mRNA Gfi1* insignificantly differed from the control (Fig. 4).

According to literature data, the CD45RO isoform may be replaced by the original variant of CD45RA. However, as already mentioned, restimulation of cells is accompanied by formation of stable final isoforms of CD45RO [15, 25, 26]; this is facilitated by the low concentration of cytokines having a common γ-chain; on the contrary, increased concentration of these cytokines in cultures of activated memory T-cells mediate activation and proliferation of immunocompetent T-cells [14].

CONCLUSIONS

Good evidence exists that alternative splicing of the *Ptprc* gene represents a feedback mechanism for the maintenance of T-cell homeostasis. Our results need further studies and supplementations. Despite the evidence, that changes in the regulation of alternative splicing are important during immune response and can critically influence cell functions, no systemic studies determining the complete range of genes regulated at the level of alternative splicing in response to an antigen or antigenic nature, have been undertaken so far.

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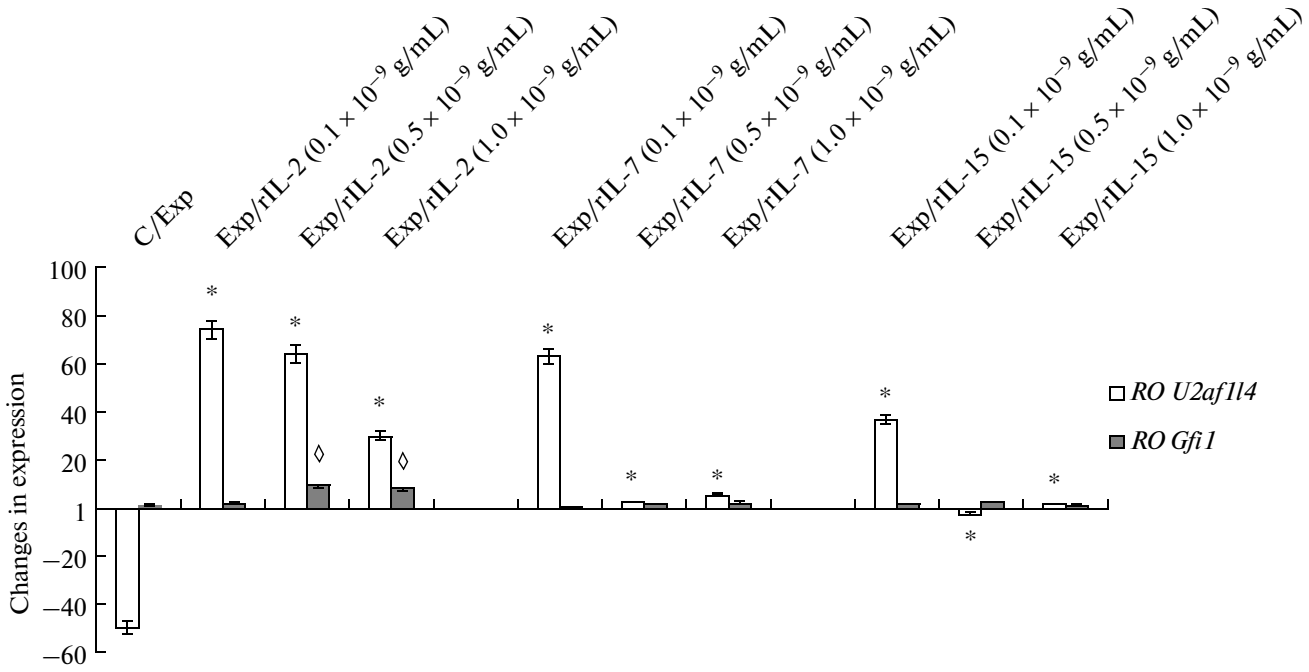

Fig. 4. Relative changes in the level of transcription of *U2af1l4* and *Gfi1* genes in CD45RO$^+$ T-cells induced by the activator and cytokines (IL-2, IL-7, IL-15) (fold change); C/Exp—the ratio of mRNA expression levels in control (untreated) samples and the samples incubated with additions of the T-cell activator (Exp); Exp IL2-0.1-0.5-1.0—the ratio of mRNA expression levels in samples incubated with additions of the T-cell activator and in samples with additions of rIL-2 0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); Exp IL7-0.1-0.5-1.0—the ratio of mRNA expression levels in samples incubated with additions of the T-cell activator and in samples with additions of rIL-7 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL); Exp IL15-0.1-0.5-1.0—the ratio of mRNA expression levels in samples incubated with additions of the T-cell activator and in samples with additions of rIL-15 (0.1×10^{-9} g/mL; 0.5×10^{-9} g/mL; 1.0×10^{-9} g/mL).

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