Expression of Regulatory T-Lymphocyte Phenotype in Human Fetal Hemopoietic and Lymphoid Cell Culture

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Cells with regulatory T-cell phenotype (Treg, $CD4^+CD25^{hi}$) were not detected in human fetal thymus, liver, bone marrow, spleen, and among blood mononuclears of 14-28-week gestation. The cells of the majority of these fetal thymuses express Treg specific marker (FOXP3 transcription marker) gene. Culturing of fetal liver and bone marrow cells on a monolayer of thymic epithelial cells induced expression of *FOXP3* gene, but induction of $CD4^+CD25^+$ membrane phenotype was detected in only 1 of 8 studied cultures (in liver and bone marrow cells). Induction of Treg differentiation is to a greater extent determined by the characteristics of hemopoietic organ cells than of thymic epithelial cells.

Key Words: *regulatory T cells; thymic epithelium; fetal hemopoietic organs*

Various regulatory T cells attract special attention of immunologists in recent years. T cells with $CD4^+CD25^{hi}$ phenotype, usually denoted as Treg, are most interesting [2,3,12,13]. Among markers of these cells (CTLA-4, GITR, *etc.*) the most specific is transcription factor (FOXP3 gene product) [7,8]. The content of Treg in the blood and lymphoid organs is low: about 5% T cells in the thymus and peripheral T-lymphocyte population (2-3% $CD4^+$ T cells) [4,12]. The targets of Treg are $CD4^+CD25^-$ and $CD8^+$ T cells: they suppress the proliferation of activated T cells and their cytokine secretion. The suppressor effect is realized through contact interaction between the target cells and Treg and secretion of suppressor cytokines interleukin-10 (IL-10) and transforming growth factor by Treg [12,14].

Treg are differentiated in the thymus from lymphoid precursors [7,10] and can form from mature $CD4^+CD25^-$-cells in the peripheral compartment of the immune system [9,16]. The main feature of their development in the thymus is that they escape negative selection, and therefore the affinity of their TCR receptor to autoantigens is higher than in other T cells [7]. Transformation of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Treg was reproduced *in vitro* not once, but the process of their *de novo* differentiation in the thymus was never reproduced *in vitro*. The development of methods for *in vitro* induction and culturing of Treg is an important practical problem, because the use of these cells for immunocytotherapy of autoimmune and allergic diseases is now discussed [5,11].

We evaluated the presence of Treg in human fetal hemopoietic and lymphoid organs, starting from the period when these cells first appeared (13-14 weeks of development [6]) and the possibility of inducing their *in vitro* differentiation by co-culturing hemopoietic cells with thymic epithelium.

MATERIALS AND METHODS

Cell sources and culturing. Human fetal (14-28-week gestation) thymus, spleen, bone marrow, liver, and blood mononuclears were used. The cells iso-

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TABLE 1. Nucleotide sequences of *FOXP3* and *GAPD* primers

Primers	Primer structure (5'-3' sequence)	Product size, n. p.
FOXP3_1 [15]	L: GAAACAGCACATTCCCAGAGTTC	
	R: ATGGCCCAGCGGATGAG	100
FOXP3_2	L: CATGATCAGCCTCACACCAC	
	R: CCACTTGCAGACACCATTTG	223
GAPD	L: ACCACAGTCCATGCCATCAC	
	R: TCCACCACCCTGTTGCTGTA	452

lated from fetal organs were washed three times in buffered saline (BS) (pH 7.4), resuspended in RPMI-1640 with 10% fetal calf serum (FCS), and cultured for 24 h in monoculture or on a primary monolayer culture of thymic epithelial cells (TEC) [1] containing about 60% keratin-positive cells (detected by FITC-labeled monoclonal antibodies to keratins 8/18, CAM 5.2; Becton Dickinson). The cells were cultured in plastic 24-mm Petri dishes (Costar) at 37°C and 5% CO_2 in RPMI-1640 (Flow) with 10% FCS (Sigma), L-glutamine (300 μg/ml, Flow), HEPES buffer (0.02 M, Serva), and gentamicin (100 μg/ml, Farmakhim). The initial concentration was 10^6 cell/ml. Experiments with each sample were repeated three times.

Cytofluorometric evaluation of membrane antigen expression. The expression of molecules on the cell surface was evaluated by flow cytofluorometry with monoclonal antibodies. FITC-Labeled monoclonal antibodies to CD3, CD8, and CD25 and monoclonal antibodies to CD4 labeled with phycoerythrin (PE; Sorben-Service) were used. In order to evaluate antigen expression, the cells were washed in medium 199 with 0.1% NaN_3 and incubated with labeled monoclonal antibodies for 30 min at 37°C. The expression of membrane antigens was evaluated on a FACSCalibur cytofluorometer (Becton Dickinson) using CellQuest 3.1 Software. The cells (10,000 cells/sample) were analyzed using an argon laser (15 mW, 488 nm) at a flow rate of 6000 cell/sec.

Polymerase chain reaction (PCR). For isolation of RNA, the cells (2×10^6 cells/tube) were washed and lyzed with 400 μl TRIzol, 200 μl chloroform was added, the mixture was intensely stirred, and centrifuged at 4°C for 10 min at 12,000*g*. An equal volume of isopropanol was added to the fraction above the protein ring (200 μl) and mixed. For RNA precipitation the samples were centrifuged at 4°C for 10 min at 12,000*g*; 80% ethanol (400 μl) was added to washed precipitate and centrifuged at 4°C for 10 min at 12,000*g*. The precipitate was dissolved in 10 μl H_2O pretreated by diethylpyrocarbonate (DEPC) and containing 25 U placental RNase inhibitor. The samples were frozen at -70°C and stored until reverse transcription reaction.

Reverse transcription was carried out on a TP-4PCR-01 Tertsik multichannel DNA amplifier (DNA-Tekhnologiya) in microtubes. The reaction mixture contained buffer for reverse transcription, 0.5 mM deoxynucleotide triphosphates, 2 μM poly-T primer, 1 mM dithiotreitol, 25 U RNase inhibitor, 200 U reverse transcriptase, and DEPC-H_2O. RNA (10 μl) was added into each tube. The reaction was carried out at 40°C for 40 min. After transcription the samples were heated at 72°C for 10 min for inactivation of reverse transcriptase. The resultant samples were stored at -20°C until PCR.

PCR was carried out on a TP-4PCR-01 Tertsik multichannel DNA amplifier (DNA-Tekhnologiya) with a reaction volume of 25 μl. PCR mixture con-

TABLE 2. Manifestation of Signs Characterizing Treg in Human Fetal Organs at Weeks 14-28 of Development

Parameter	Simultaneous expression of CD4 and CD25, %			Expression of *FOXP3*, % of positive results
	M±*m*	range	*n*	
Thymus	1.28±0.85	0.1-2.9	19	8/15
Liver	0.99±0.43	0-3.7	9	0/7
Bone marrow	0.86±0.41	0.1-3.3	9	0/7
Blood mononuclears	0.1	0-0.3	3	0/3

Note. *Numerator: number of cases with *FOXP3* expression; denominator: total number of studied suspensions.

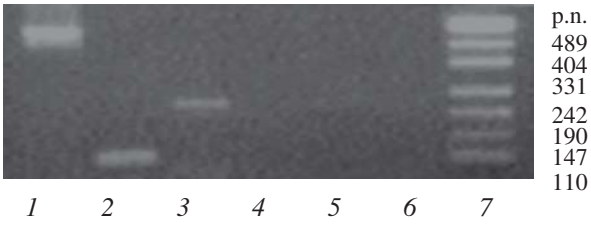

Fig. 1. Expression of *FOXP3* mRNA by cells of different organs of a 19-week fetus. *1*) expression of *GAPD* mRNA: product length 452 n. p. (control); *2*) expression of *FOXP3_1* mRNA by thymocytes: product length 100 n. p.; *3*) expression of *FOXP3_2* mRNA by thymocytes: product length 223 n. p.; *4*) no expression of *FOXP3_1* mRNA by liver cells; *5*) no expression of *FOXP3_1* mRNA by splenic cells; *6*) no expression of *FOXP3_1* mRNA by bone marrow cells; *7*) marker DNA.

tained PCR buffer, 0.2 mM dNTP (dinucleotide triphosphate), 0.5 pmol/μl FOXP3 or control primers for glyceraldehyde-3-phosphate dehydrogenase (GAPD), and 2.5 U recombinant thermostable DNA polymerase (Table 1). The sample obtained by reverse transcription (2 μl) was added to the mixture. The conditions of the reaction were as follows: 5 min at 94°C (cycle 1); 36 cycles: 30 sec at 94°C, 1 min at 53°C, and 30 sec at 72°C. The samples were stored at -20°C.

Amplification products were analyzed by electrophoresis in 2% agarose gel. Tris-borate buffer (TBE) was added (30 ml) to 0.6 g agarose and heated until large bubbles appeared. The mixture was then cooled to 60°C, 4 μl 1% ethidium bromide solution was added, and the cuvette for gel was filled. The samples were mixed with the buffer for samples in 2:1 ratio and applied to the gel. Molecular weight markers were used in each test. Electrophoresis was carried out in TBE buffer at 100 V for 1 h and the results were then evaluated on a

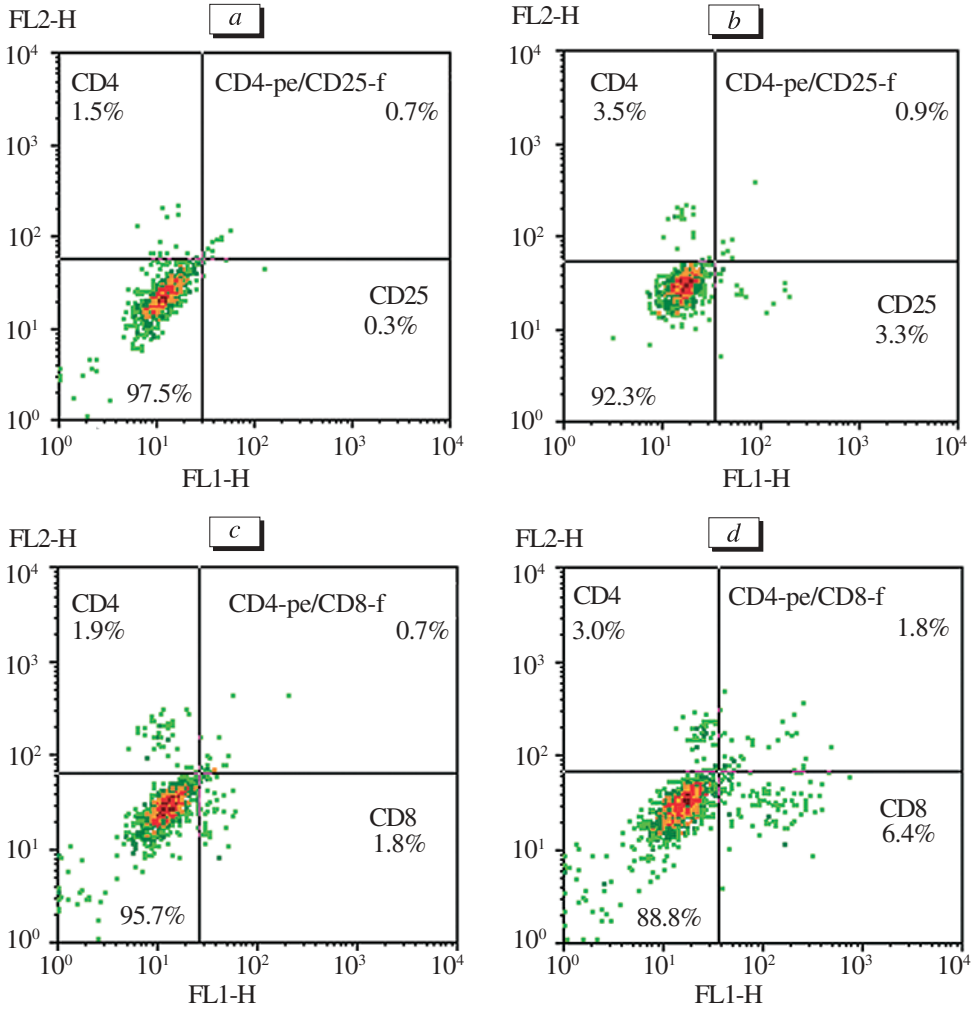

Fig. 2. Cytometric parameters of the results of co-culturing of fetal liver cells and thymic epithelial cells (TEC). Differentiation of T cells and the absence of Treg differentiation. *a*, *c*) hepatocyte monocultures; *b*, *d*) co-culturing of liver cells and TEC. *a*, *b*) determination of $CD4^+$ (by ordinates) and of $CD8^+$ cells (by abscissas); *c*, *d*) determination of $CD4^+$ (by ordinates) and of $CD25^+$ cells (by abscissas). $CD4^+CD25^+$ cells are located in the right upper quadrant. Abscissa: intensity of green fluorescence; ordinates: intensity of orange fluorescence.

transilluminator. The results were processed using Gel Imager videosystem, images using Photoshop 6.0 software.

RESULTS

Detection of human fetal organ Treg. Suspensions of human fetal (14-28-week gestation) thymus, liver, spleen, bone marrow cells and blood mononuclears were studied by flow cytofluorometry. The fraction of lymphoid cells was distinguished on histograms by direct and angular light scattering. The content of $CD4^+CD25^+$ cells in these populations was evaluated by two-color flow cytofluorometry (Table 2). There were virtually no $CD4^+CD25^+$ cells in all suspensions. It is doubtful that detection of 3-3.5% cells of this phenotype in solitary samples of liver and bone marrow cells can be enterpreted as the presence of Treg in them.

On the other hand, expression of *FOXP3* was detected in 50% studied thymocyte samples (Fig. 1), but not in fetal hepatic, bone marrow, splenic or blood cell samples. Expression of FOXP3 is acknowledged to be the most specific indicator of Treg differentiation. Hence, the presence of Treg in fetal thymocytes can be detected by *FOXP3* gene expression, but not by the presence of characteristic membrane phenotype of these cells. Presumably, these cells are not quite mature. It will be interesting to evaluate functional activity of Treg isolated from the thymus at different stages of intrauterine development.

Induction of Treg phenotype in fetal hemopoietic organ cell cultures. Co-culturing of liver and bone marrow cells from fetuses of 14-28-week gestation on a TEC monolayer increased the content of $CD4^+$ and $CD8^+$ cells, that is, induced the differentiation of thymocytes, this indicating func-

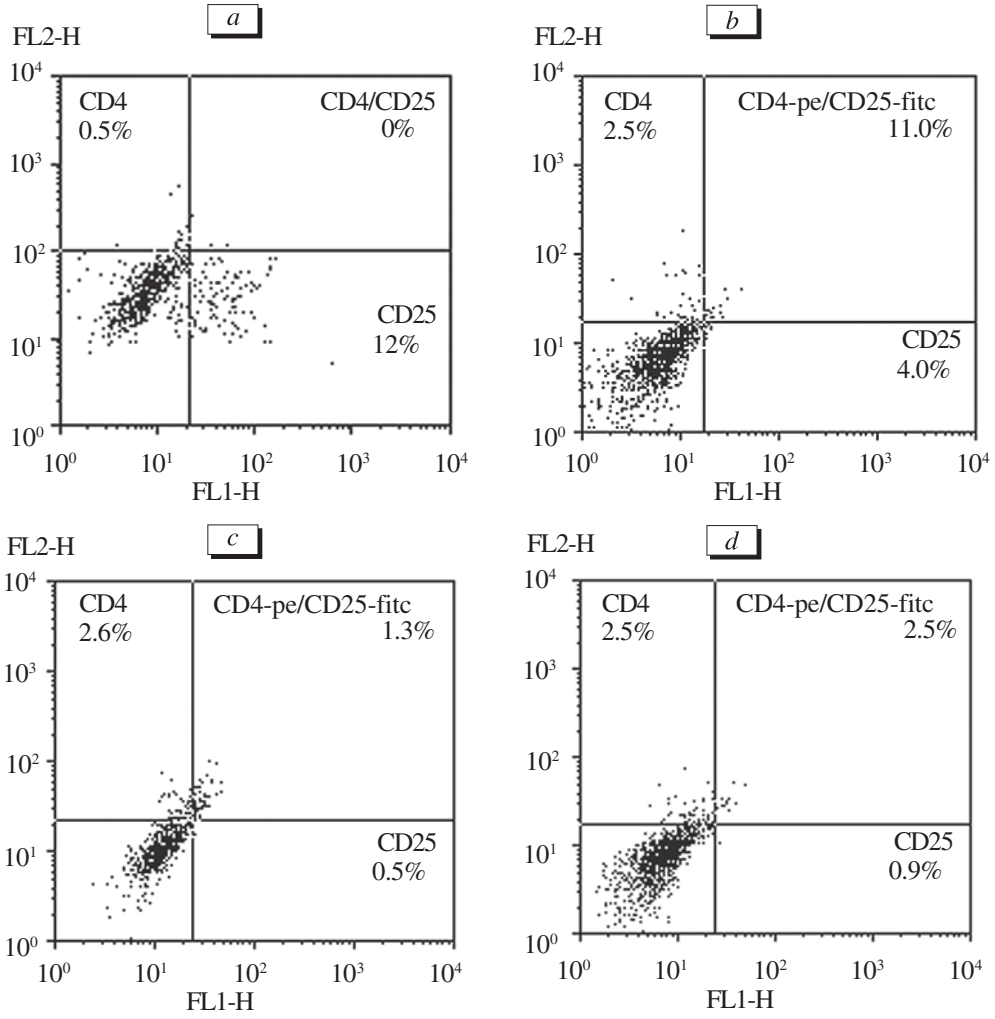

Fig. 3. Cytometric parameters of the results of co-culturing of two fetal liver cell samples with TEC. Presence (*a*, *b*) and absence (*c*, *d*) of Treg differentiation. *a*, *c*) hepatocyte monocultures from two different fetuses; *b*, *d*) co-culturing of hepatocytes and TEC. Here and in Fig. 4: abscissas: intensity of green fluorescence (binding of FITC-labeled antibodies to CD25); ordinates: intensity of orange fluorescence (binding of phycoerythrin-labeled antibodies to CD4). Cells of $CD4^+CD25^+$ phenotype are in the right upper quadrant.

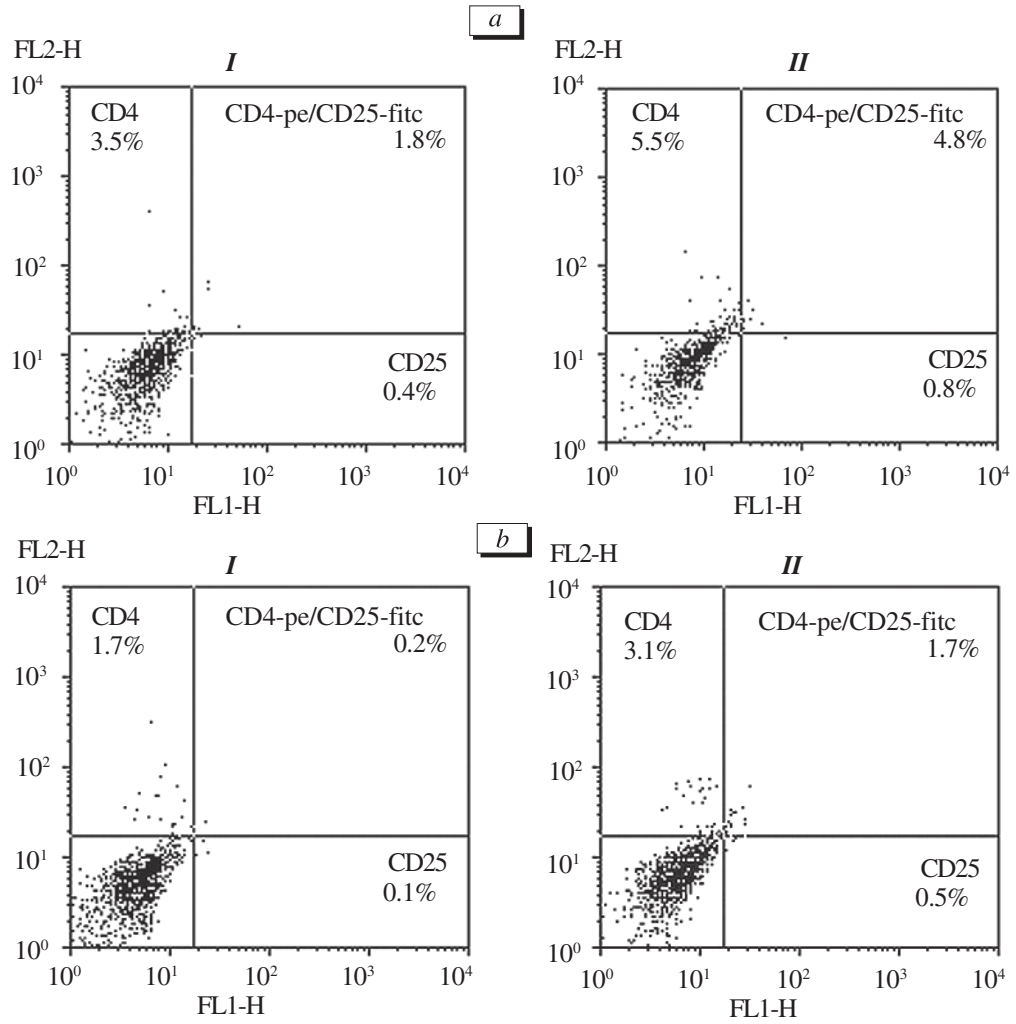

Fig. 4. Cytometric parameters of the results of co-culturing of two samples (*a*, *b*) of fetal bone marrow cells with TEC. Presence (*a*) and absence (*b*) of Treg differentiation. *I*) bone marrow cell monocultures from two different fetuses; *II*) co-culturing of bone marrow cells and TEC.

tional intactness of TEC. However, no cells with phenotype characteristic of Treg ($CD4^+CD25^+$) appeared (Fig. 2). This was characteristic of 7 of the 8 studied co-cultures of fetal hemopoietic cells (liver and bone marrow) and TEC.

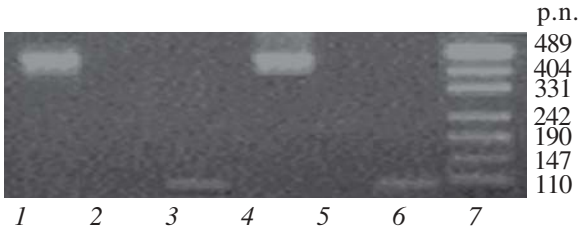

Fig. 5. Expression of *FOXP3_1* mRNA by liver and bone marrow cells co-cultured with TEC. *1*, *4*) expression of *GAPD* mRNA: product length 452 n. p. (control); *2*) no expression of *FOXP3_1* mRNA by liver cells; *3*) expression of *FOXP3_1* mRNA by liver cells co-cultured with TEC: product length is 100 n. p.; *5*) no expression of *FOXP3_1* mRNA by bone marrow cells; *6*) expression of *FOXP3_1* mRNA by liver cells co-cultured with TEC: product length is 100 n. p.; *7*) marker DNA.

However, in one case clear-cut signs of $CD4^+CD25^+$ cell differentiation were observed in liver and bone marrow cell cultures during their co-culturing with TEC from the same source (Fig. 3, *a*, *b* and 4, *a*). The induction was most pronounced in liver cell culture: $CD4^+CD25^+$ cells were virtually completely absent in monoculture (Fig. 3, *a*), but their number increased to 11% after co-culturing with TEC (Fig. 3, *b*). This value 2-fold surpassed the content of $CD4^+CD25^+$ cells in adult human thymus [12] and in fetal thymus of the same term [6]. The degree of $CD4^+CD25^+$ phenotype induction in bone marrow cell culture was significantly less pronounced (the number of cells with this phenotype increased approximately 3-fold) and reached 5% of total cell count in lymphoid organs of human fetuses and adults [12]. Induction of $CD4^+CD25^+$ phenotype was paralleled by expression of *FOXP3* gene (Fig. 5).

Co-culturing of liver and bone marrow cells from another fetus of the same gestation term with

the same TEC led to approximately 2-fold increase in the percentage of $CD4^+CD25^+$ cells (2.5% for liver cells and 1.7% for bone marrow cells; Fig. 3, *c*, *d* and 4, *b*). This result is regarded as negative. Hence, the efficiency of induction of $CD4^+CD25^+$ cells is determined not by the differentiating effect of TEC, but by the characteristics of hemopoietic cells, because the positive result was observed in the liver and bone marrow cell cultures derived from the same fetus, while the same TEC induced differentiation in one series of cultures and did not induce it in another series.

On the other hand, $CD4^+CD25^+$ phenotype induced in co-culture with TEC did not quite correspond to classical human Treg phenotype ($CD4^+CD25^{hi}$), because the level of CD25 expression was low. Presumably, this also should be regarded as an indicator of incomplete maturing of these cells, whose functional activity in this case is to be once more confirmed, too.

Summing up these results, we conclude that human fetal (14-28-week gestation) hemopoietic and lymphoid organs contain no cells with the classical membrane Treg phenotype ($CD4^+CD25^{hi}$). On the other hand, cells expressing *FOXP3* are present in fetal thymus. *In vitro* co-culturing with TEC in some cases leads to induction of membrane Treg phenotype and *FOXP3* expression in fetal liver and bone marrow cells. Induced $FOXP3^+$ cells differ from the classical human Treg cells by weak expression of CD25, which requires validation of their functional activity as suppressor cells. Nonetheless, these findings confirm the possibility of reproducing Treg differentiation and hemopoietic precursor cells *in vitro* in cultures with TEC.

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