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Transcription Factors NFAT2 and Egr1 Cooperatively Regulate the Maturation of T-Lymphoma *in vitro*

E. K. Koltsova[1,2]*, D. L. Wiest[2], and T. P. Vavilova[1]

[1]*Moscow State Medical Stomatological University, Delegatskaya ul. 20/1, 127473 Moscow, Russia; E-mail: ekoltsova@gmail.com*
[2]*Immunobiology Working Group, Division of Basic Science, Fox Chase Cancer Center, Philadelphia 19111, USA*

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Abstract—We have demonstrated that transcription factors Egr1 and NFAT2 cooperate in regulation of the early stages of T-lymphocyte development, whereas the related factors Egr2 and Egr3 do not cooperate with NFAT2. Egr1 and NFAT2 are shown to cooperatively control gene expression of the regulatory factor Id3 and recombinase Rag2, whose functions are critical for T-lymphocyte differentiation. Thus, the concerted action of the transcription factors Egr1 and NFAT2 can play a crucial role in regulation of the T cell differentiation *in vitro* due to the cooperative regulation of *Id3* and *Rag2* gene expression.

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Key words: β-selection, Egr, NFAT, pre-TCR, T-lymphocytes

The development of T cells is an important and ceaseless process in the immune system of vertebrates. In the initial stages, precursor cells migrate from the bone marrow into the thymus. During the differentiation, immature T cells undergo a number of proliferation cycles and also rigorous selection, which result in production of mature T-lymphocytes [1].

The differentiation of T cell can be subdivided into separate stages (DN I, DN II, DN III, DN IV, DP, SP) characterized by expression of surface marker molecules [2] (Fig. 1a). There are three crucial checkpoints that are required for effective development of T cells (β-selection, negative and positive selection). During the first stage of the thymocyte development, the β-chain gene of the T cell receptor (TCR) is rearranged and the so-called pre-TCR is formed.

A signal transduced via the pre-TCR induces a rearrangement in genes of the TCR α-chain locus, which results in generation of a functional α-chain. Moreover, the pre-TCR activation regulates the differentiation program through activation of various transcription factors, such as NF-κB, Egr, NFAT, E-proteins, β-catenin/TCF-1, Myb, and GATA-3 [3-11]. The hierarchy and order of the interaction of these transcription factors are insufficiently studied.

Egr proteins are expressed by many cell types and regulate diverse functions of cells. Thus, Egr proteins are activated in response to pre-TCR signals and play an important role in regulation of the TCR α-chain rearrangement. Functions of Egr proteins are overlapping and degenerate, so that genetic knockout of each of these genes in mice does not dramatically destroy T-lymphocyte development [12, 13]. However, inhibition of activities of all Egr proteins completely stops the development in the β-selection stage [14]. Moreover, Egr-protein overexpression promotes the β-selection of T-lymphocytes even in the absence of signals from the pre-TCR [14-16]. Egr proteins consist of a number of domains and are capable of complexing with other transcription factors. Although Egr proteins play an important role in T-lymphocyte development, in particular in β-selection, their interactions with other signaling pathways and different regulators of gene expression are still unclear.

The NFAT family proteins also are transcription factors, which are, in particular, activated in response to pre-TCR signals and also play an important role during β-selection [4]. Using knockout mice, it has been shown that NFAT-proteins perform important and overlapping functions during T-lymphocyte differentiation [17-22].

Abbreviations: DN) double negative; DP) double positive; Egr) early growth response; FACS) fluorescence activated cell sorting; NFAT) nuclear factor of activated T cells; SP) single positive; TCR) T cell receptor; pre-TCR) pre-T cell receptor.

* To whom correspondence should be addressed.

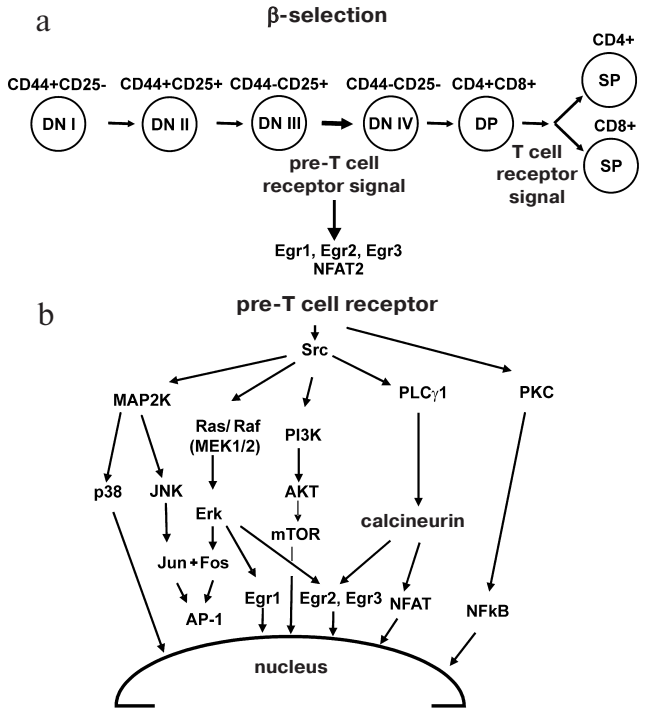

Fig. 1. Stages of T-lymphocyte development (a) and the scheme of signal transduction from the pre-T cell receptor (b).

Thus, NFAT2 plays the most important role in regulation of the development of T cells from double negative (DN) to double positive (DP) [18, 22]. Moreover, Egr1- and NFAT-proteins can cooperate in regulation of expression of various genes, such as *IL-2*, *TNF*, *CD154*, and *FasL* [23-26].

We supposed that the Egr1 and NFAT signaling pathways should interact in thymocytes and demonstrated that Egr1 and NFAT2 cooperated in regulation of β-selection. Thus, an enhanced expression of Egr1 together with NFAT2 in the Scid.adh cell line (taken as a suitable model of the intrathymus T cell development) considerably down-regulated the expression of CD25 (the β-selection marker). We have also shown a cooperation of Egr1 and NFAT2 in the regulation of expression of the *Id3* and *Rag2* genes, which play the most significant role during the early stages of T-lymphocyte development. Thus, based on our findings, Egr1 and NFAT2 are suggested to co-regulate β-selection and subsequent differentiation of early T-lymphoma *in vitro*.

MATERIALS AND METHODS

Cell cultures. The Scid.adh cell line expresses a chimeric receptor TAC:CD3ε consisting of an extracellular domain of the human IL-2(CD25) receptor α-chain and the cytoplasmic region of the CD3 receptor ε-chain which is a component of the T cell receptor complex [27]. The Scid.adh cells were cultured in Iscove complete DMEM medium containing 10% fetal calf serum, antibiotics (penicillin/streptomycin, 100 U/ml each), L-glutamine (2 mM), essential amino acids (0.1 mM), and β-mercaptoethanol [14]. The cells were incubated at 37°C in the presence of 5% CO_2. To stimulate the cells, we used mouse monoclonal antibodies (10 μg/ml) to the chimeric receptor TAC. Antibodies were preincubated overnight in phosphate buffer saline in a 24-well plate. Then cell suspension was added for subsequent stimulation during 24 h [27]. The hybridoma (hd 245/332) producing antibodies to the chimeric receptor TAC was obtained from the American Collection of Cell Cultures, with permission of Dr. T. Waldman (National Institutes of Health, USA).

To elucidate roles of individual signaling pathways in T-lymphocyte differentiation and expression of *Egr* and *TCR-α* genes, the stimulated cultures were incubated in the presence of dimethylsulfoxide (control) or one of the following inhibitors: a calcineurin inhibitor, cyclosporin A (2 μM) [28]; a protein kinase Src inhibitor, PP2 (1 μM) [29]; a protein kinase MEK inhibitor, UO126 (5 μM) [30, 31]; a protein kinase p38 inhibitor, SB203580 (25 μM) [30]; a protein kinase JNK inhibitor, JNKII (1 μM) [32]. The most efficient but the least toxic for the cells concentrations of each inhibitor were determined by titration. The cells were cultured for 24 h, and the differentiation level was assessed measuring expression of the CD25 surface marker by flow cytometry (FACS). The cells were stained with a commercial antibody (BD PharMingen, USA) conjugated with a corresponding fluorescent dye and analyzed using a BD LSRII or FACSVantage cytometer (BD PharMingen), on staining with propidium iodide (PI) to exclude dead cells.

Plasmids. A retroviral vector MSCV-GFP-NFATc1 encoding the NFATc1 (NFAT2) full-size cDNA was described earlier [33]. cDNA of Egr1 containing on the C-end a sequence encoding the marker protein was cloned into the retroviral vector LZRS-YFP by polymerase chain reaction (PCR) according to the standard protocol. This vector contains the internal ribosomal entry site (IRES) which provides for an independent translation of two proteins: Egr1 and the marker protein (yellow fluorescent protein (YFP)). The retroviral vector LZRS-YFP was kindly provided by Dr. D. Vignali (St. Jude Children's Research Hospital, Nashville, USA).

Production and transduction of retroviruses. The packing cells Phoenix E kindly provided by Dr. G. Nolan (Stanford University, USA) were transfected with retroviral vectors using the calcium phosphate method as described in [34]. The transfection efficiency was determined evaluating percent of the GFP- or YFP-positive cells by flow cytometry (FACSVantage SE; BD Biosciences, USA). All cell lines were cultured in the complete Iscove medium as described above. The

Scid.adh–TAC:ε cells (10^6 cell/ml) were infected by centrifugation for 45 min at 30°C with the retroviral supernatant supplemented with polybrene (8 μg/ml) as described in [35]. Thirty hours after infection the cells were analyzed or sorted by flow cytometry to analyze the gene expression.

Measurement of gene expression. RNA was isolated using an RNAEasy purification kit (Qiagen, USA) and treated with DNase I (Invitrogen, USA). Then the first chain of cDNA was synthesized using a Superscript II reverse transcriptase (RT) according to the random primers Protocol (Invitrogen). Expression of the NFAT family genes was measured by RT-PCR using the following primers: NFAT1 (Fw 5′-ATCACTGGGAAAACGGTCACC-3′, Rv 5′-TTAGGCTGGCTCTTGTCTTTAATCC-3′), NFAT2 (Fw 5′-CCAAGTCTCTTTCCCCGACATC-3′, Rv 5′-TCAGCCGTCCCAATGAACAG-3′), NFAT3 (Fw 5′-GGATTACTGGCAAGATGGTGGC-3′, Rv 5′-AGTCTGGCAGGAAGTTGGAACC-3′), NFAT4 (Fw 5′-CAGGGAAAAATGTCAAGGGGC-3′, Rv 5′-CAACTGTGGCAAATGGGTGGAG-3′), Actin (Fw 5′-CCTAAGGCCAACCGTGAAAAG-3′, Rv 5′-TCTTCATGGTGCTAGGAGCCA-3′).

The reaction products were analyzed upon electrophoresis in 1% agarose gel in the presence of ethidium bromide. The gene expression was measured by real-time TaqMan-PCR using commercial synthetic primers and probes (ABI, USA). The reactions were performed thrice for each gene. The expression level of each gene was normalized relatively to the actin gene expression. The findings are presented as the ratio of expression of a specific gene in the stimulated cells to its expression in the unstimulated ones.

RESULTS

Inhibition of the calcineurin signaling pathway completely blocks T cell differentiation but has virtually no effect on Egr1 expression. Egr proteins play an important role in transduction of pre-TCR signals during β-selection (Fig. 1) [12, 14, 15, 36]. Nevertheless, transcription factors capable of controlling T cell development in cooperation with Egr proteins are still unknown. To detect them, we used the Scid.adh cell line [27]. The stimulation of these cells mediated through a chimeric receptor imitates a signal from the pre-TCR, and this decreases the surface expression of CD25 [37]. We were mainly interested in biochemical inhibitors of signaling pathways that disturb T cell differentiation but do not influence *Egr* gene expression in response to activation through the pre-TCR. Such signaling pathways can potentially functionally interact with Egr proteins, whereas the pathways concurrently suppressing both the thymocyte differentiation and Egr activation seemed to converge on the level preceding Egr proteins.

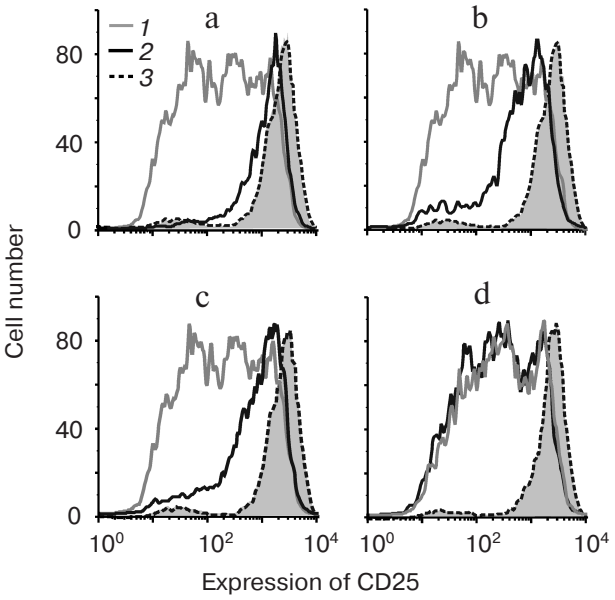

Fig. 2. Effects of inhibitors of calcineurin, protein kinases Src, Erk, and JNK (a-d, respectively) on differentiation of Scid.adh cells: *1*) anti-TAC stimulation; *2*) anti-TAC stimulation + inhibitor; *3*) unstimulated cells.

The pre-TCR activates various signaling pathways, including signaling cascades MAP2K, MEK/Erk, and PI3K/AKT, as well as the pathways with involvement of phospholipase C and protein kinase C (Fig. 1b).

The effects of different inhibitors on T-lymphocyte differentiation are shown in Fig. 2. Inhibition of protein kinases Src and Erk and of calcineurin arrested T cell development at the DN III stage and, consequently, prevented β-selection. But inhibition of protein kinase JNK (Fig. 2) and protein kinases C, p38, PI3K, mTOR, and phospholipase Cγ1 (data not presented) did not affect T cell differentiation. Thus, signals mediated through calcineurin, Src, and Erk are important for regulation of β-selection.

To elucidate the influence of signaling pathway inhibition on expression of the genes required for β-selection, we have analyzed changes in expression of the Egr family genes (*Egr1*, *Egr2*, *Egr3*) and also of the TCR α-chain gene (*TCR-α*) using real-time PCR. Expression of these genes was determined in the Scid.adh cells stimulated with antibodies to the TAC chimeric receptor and treated with biochemical inhibitors. Note that among inhibitors affecting the differentiation, only the calcineurin inhibitor cyclosporin A partially inhibited expression of the gene of an early response protein Egr1. However, such inhibitors as UO126 (the MEK/Erk-signaling pathway) and PP2 (an inhibitor of protein kinase Src) also suppressed the further differentiation of T-lymphocytes and expression of all Egr family genes and of *TCR-α* (Fig. 3). Strong inhibition of protein kinase Src seems to be caused by its location on the top of many signaling cascades (Fig. 1b).

The inhibition of the calcineurin–NFAT signaling pathway completely blocked the differentiation but only partially inhibited the *Egr1* gene expression; therefore, *Egr1* could not be a target gene for this signaling pathway and, consequently, could cooperate with NFAT in regulation of the early stages of T cell development.

Egr1 and NFAT cooperate in regulation of β-selection. Calcineurin regulates the activity of the transcription factor NFAT, which plays an important role in T-lymphocyte development. The Scid.adh cell line was shown by RT-PCR to express NFAT1, NFAT2, and NFAT4 (data not presented). Of these factors, only NFAT2 is involved in regulation of differentiation of DN T-lymphocytes to DP cells [18, 22]. Therefore, to elucidate whether Egr1 could interact with NFAT2, we expressed both Egr1 and NFAT2 in the Scid.adh cell line using retroviral vectors with different fluorescent markers (YFP and GFP). The packing Phoenix E cell line was transfected with a retroviral vector encoding the full-size cDNA of Egr or NFAT and the fluorescent protein. The retrovirus production, as well as expression of each Egr protein or NFAT2, was assessed using flow cytometry by expression of the fluorescent markers YFP and GFP, respectively (Fig. 4a). Usually, the transfection efficiency of the Phoenix cells was 20-30%. This allowed us to prepare the desired viral supernatant with the virus concentration sufficient for infecting the Scid.adh cells. Then Egr1-YFP, Egr2-YFP or Egr3-YFP, and NFAT-GFP were co-transduced into the Scid.adh cell line as components of the corresponding retroviruses (Fig. 4b). Note, that the co-expression of two transcription factors resulted in a markedly more pronounced decrease in the CD25 expression than their separate expression (Fig. 5a). Consequently, the transcription factors Egr1 and NFAT2 can cooperate in regulation of T-lymphocyte β-selection *in vitro*.

No cooperative action was observed when NFAT2 was co-expressed with Egr2 or Egr3 (Fig. 5, b and c). However, Egr2 or Egr3 overexpression itself caused T-lymphoma differentiation significantly stronger than the overexpression of Egr1.

NFAT2 and Egr1 cooperatively regulate gene expression. To understand the molecular mechanism of the cooperative action of the transcription factors Egr1 and NFAT2, we decided to identify target genes whose expression was cooperatively regulated by these transcription factors. First of all, we paid attention to the genes the expression of which had been earlier shown to change during β-selection and whose functions were important during this stage. It was interesting to reveal the *Rag2* and *Id3* genes among the genes analyzed. Rag2 is an enzyme that catalyzes recombination of the TCR α- and β-chain genes [38]. The regulatory factor Id3, in turn, suppresses activities of E-proteins, which is essential for β-selection [39]. Thus, functions of these two genes are extremely important for regulation of transition from the DN III to the DN IV stage. We found that the *Rag2* expression decreased much more strongly in the presence of both Egr1 and NFAT2 than in the presence of each factor alone. However, the expression of the *Id3* gene was higher in the presence of the two factors (Fig. 6a). The activation of the gene *Id3* expression or the gene *Rag2* suppression in the concurrent presence of both Egr1 and NFAT2 was greater than the sum of the individual Egr1 and NFAT2 effects ($p < 0.05$).

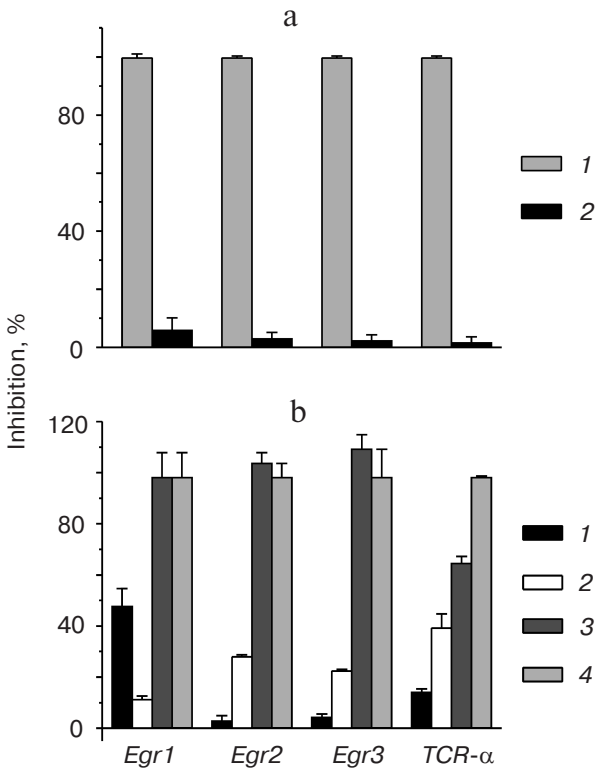

Fig. 3. Effects of inhibition of signaling cascade components on expression of target genes. a) Inhibition of Scr completely blocks expression of the *Egr* and *TCR-α* genes: *1*) control; *2*) PP2. b) Cyclosporin A only partially inhibits expression of *Egr1*: *1-4*) CsA, UO126, JNKII, and control, respectively.

The activation of *Egr1* gene expression in the presence of the Egr1 and NFAT complex (Fig. 6b) was a rather interesting observation, which could be explained by existence of the so-called feedback loop. A complex consisting of Egr1 and NFAT2 seemed also to markedly activate expression of the *Egr1* gene. But this hypothesis has to be additionally tested. No significant changes were observed in the *Egr2* and *Egr3* gene expression in response to co-expression of Egr1 and NFAT2 (Fig. 6b).

DISCUSSION

The pre-TCR activates a number of signaling cascades, which regulate the early stages of T-lymphocyte

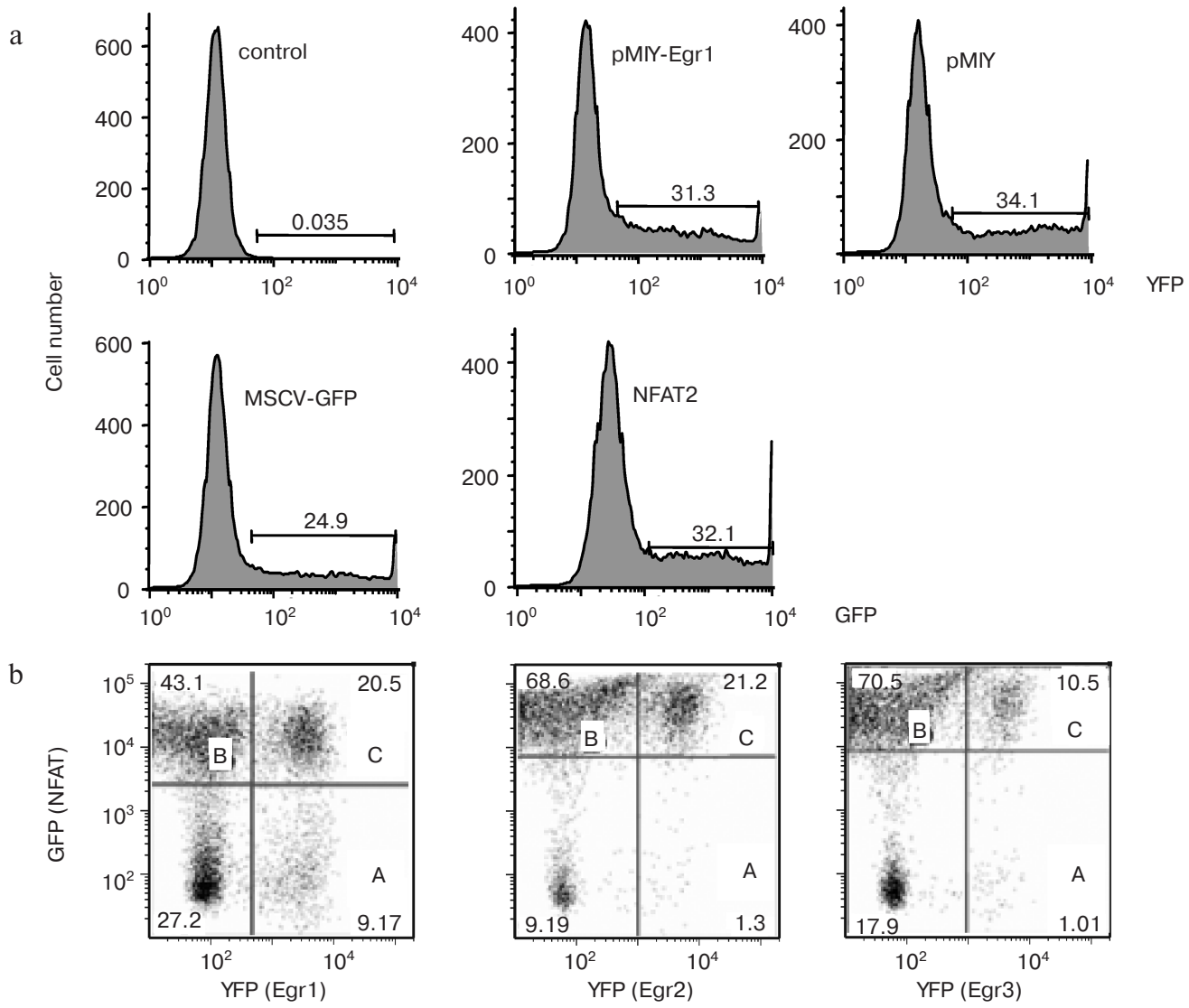

Fig. 4. Production (a) and co-expression (b) of retroviruses encoding Egr1 or NFAT. A-C, Egr (YFP^+), NFAT (GFP^+), and both Egr/NFAT proteins (GFP^+/YFP^+), respectively.

differentiation including β-selection. Interaction of transcription factors and their cooperative or individual binding with promoter regions of DNA seems to be a possible mechanism of integrating information from different signaling pathways. The cooperation between NFAT and the transcription factor AP-1 [40] or between NFAT and FoxP3 [41] exemplify such an interaction. However, it is difficult to identify particular molecules responsible for integration of the intracellular signal. In the Scid.adh cell line, β-selection is regulated by Egr1, and on this model we succeeded in detecting a factor which regulates the early stages of T cell development in cooperation with Egr1. This factor is NFAT2.

Functions of Egr proteins were earlier shown to be necessary for β-selection of thymocytes and their further development [12, 14, 15]. Mice genetically deficient in any *Egr* genes manifested no disorders during the early stages of T-lymphocyte development. However, dominant negative mutants with affected functions of all Egr proteins completely inhibited β-selection, suggesting the existence of overlapping functions within the Egr family [12, 13]. Moreover, overexpression of any of the Egr proteins, including Egr1, which virtually replaced a pre-TCR signal, was sufficient for transition from the DN III to the DN IV stage [14, 15]. Nevertheless, up to now the functional interaction of Egr proteins with other transcription factors during regulation of the early T cell differentiation has not been characterized in detail.

The NFAT family members, in particular NFAT2, are important effector molecules in the signal transduction from the pre-TCR [4]. The genetic knockout of the NFAT2 gene was shown to disrupt the DN III to DN IV

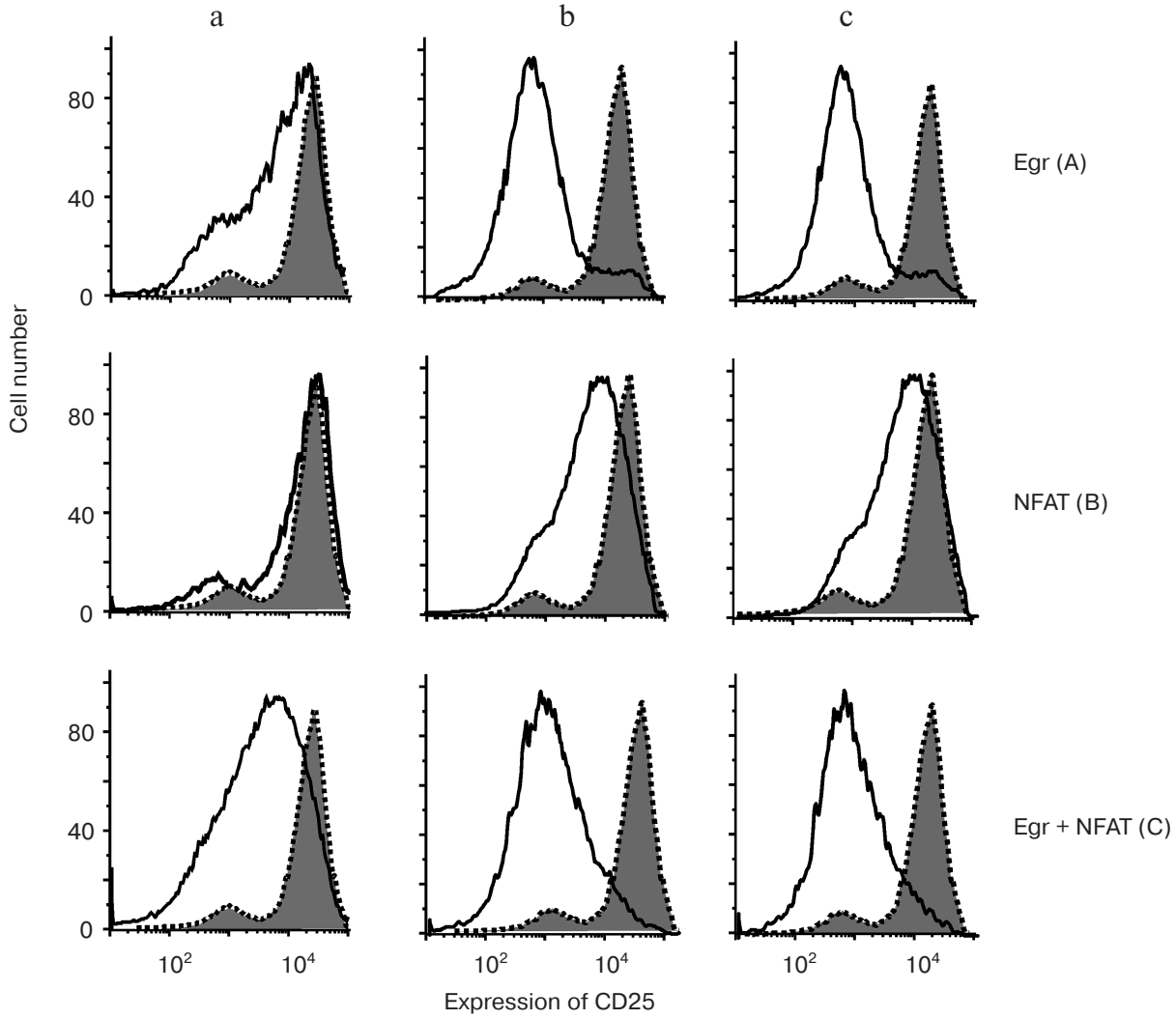

Fig. 5. Effects of the transcription factors Egr, NFAT2, and Egr-NFAT2 (A-C, respectively, in Fig. 4) on Scid.adh cell differentiation: a) Egr1; b) Egr2; c) Egr3. Thick curves present the transfected cells, and dotted lines show the controls.

transition [18, 22]. Moreover, it was shown that NFAT2 in cooperation with Egr1 regulated expression of such immunologically important genes as *IL-2*, *TNF-α*, *CD154*, and matrix metalloproteinase *MT1-MMP* [23, 26, 42, 43]. We have shown that co-expression of Egr1 and NFAT2 accelerates the differentiation of the early T-cell lymphoma *in vitro* significantly stronger than a separate expression of any of these factors. We have also shown that regulation of the β-selection and T cell differentiation is based on the interaction of Egr1 and NFAT2 and their cooperative influence on expression of the *Id3* and *Rag2* genes.

And what is a molecular basis of the cooperative effect of Egr1 and NFAT2? Based on the available data, several possible mechanisms can be supposed. Thus, a physical interaction of Egr1 with NFAT2 has been shown, and this complex can activate *IL-2* and *TNF* [23]. Moreover, NFAT2 can also bind with the transcription factor FOXP3, and this complex can regulate the CD25 gene promoter activity [41]. Secondly, Egr1 and NFAT can also bind with composite sites recognizable only by a certain complex of transcription factors. In particular, such is the regulation of the FasL expression [44]. The third example is presented by regulation of the *CD154* gene expression via binding different sites located closely to one another [26]. Owing to diversity of target genes whose expression is regulated by NFAT and Egr1, it is possible that there are several alternative mechanisms of the cooperative action of these factors during T cell differentiation.

Thus, it has been shown that the effects of two transcription factors involve their cooperative regulation of expression of the *Id3* and *Rag2* genes products, which are essential for β-selection. Id3 is an HLH-factor that forms heterodimers with E-proteins HEB and E2A, preventing their binding with DNA [39, 45]. Inhibition of the E-protein activities is also important during β-selection. It

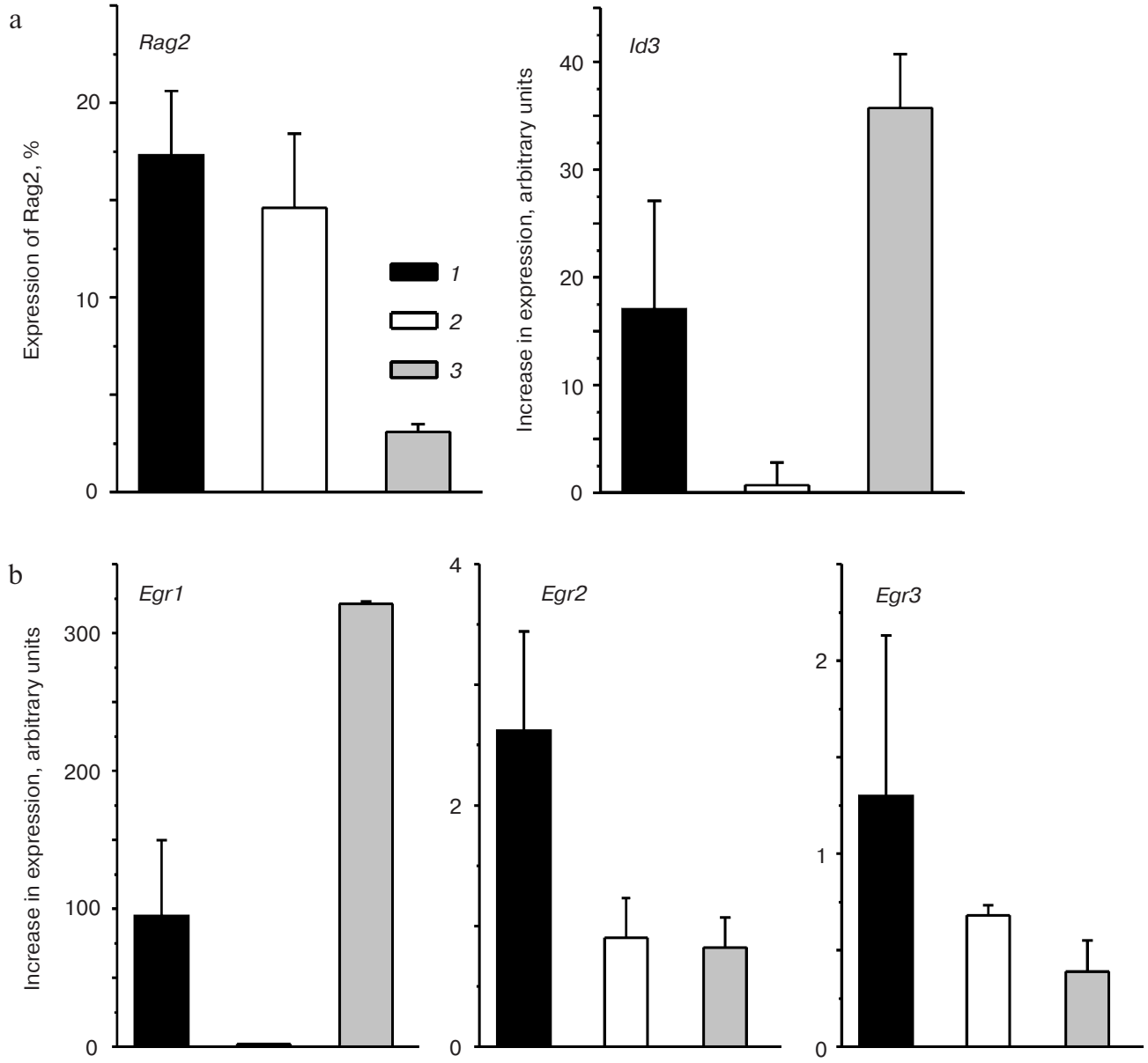

Fig. 6. Effects of transcription factors Egr1 and NFAT2 on expression of *Rag2* and *Id3* (a) and *Egr1*, *Egr2*, and *Egr3* genes (b): *1-3*) Egr1/YFP, NFAT/GFP, and Egr1–NFAT/YFP–GFP, respectively.

seems that Id3 and the related products are essential for regulation of the early thymocyte differentiation. Activation of the *Rag* genes is necessary for rearrangement of the TCR α- and β-chain genes [38]. Upon the successful completion of the TCR genes, the *Rag* gene expression sharply decreases.

Because *Id3* occurred to be an important target gene for Egr1 and NFAT2, we performed a computerized analysis of the gene *Id3* promoter sequence based on the data on this promoter regulation in myoblasts [46]. We have not detected in it composite binding sites of Egr1 and NFAT2 or independent binding sites of NFAT. Nevertheless, the promoter contained conservative Egr1 sites; therefore, a cooperative activation of Id3 via the Egr1 and NFAT2 binding with the composite or adjacent sites was unlikely. Possibly, the *Id3* expression in thymocytes is regulated with involvement of an unidentified signaling molecule, which is activated under conditions of Egr1 and NFAT2 co-expression.

Egr1 and NFAT2 are independently regulated by Erk-MAPK and calcineurin signaling cascades. In turn, the Egr1 and NFAT2 interaction during β-selection can provide a mechanism for integration of information from these two signaling pathways.

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