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Evaluation of mRNA Expressions of *TOX* and *NR4A*s in CD8$^+$ T cells in Acute Leukemia

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ABSTRACT

Background: Thymocyte selection-associated high mobility group box protein (TOX) and members of the nuclear receptor 4A (NR4A) are known as transcription factors involved in T cell exhaustion.

Objective: To evaluate the mRNA expression of *TOX* and *NR4A1-3* in CD8$^+$ T cells in acute leukemia.

Methods: Blood samples were obtained from 21 ALL and 6 AML patients as well as 20 control subjects. CD8$^+$ T cells were isolated using MACS. Relative gene expression of *TOX* and *NR4A1-3* was then evaluated using qRT-PCR.

Results: Comparison of mRNA expression of *TOX* in CD8$^+$ T cells showed no significant difference among the study groups (p>0.05), while the expression of *NR4A1* was significantly lower in AML patients than in the control group (p=0.0006). Also, the expression of *NR4A2* and *NR4A3* was significantly lower in both ALL (p=0.0049 and p=0.0005, respectively) and AML (p=0.0019 and p=0.0055, respectively) patients.

Conclusion: *NR4A*s expressions were found to be lower in CD8$^+$ T cells from patients with AML and ALL compared to controls, whereas the mRNA expression of *TOX* showed no significant difference. Although TOX and NR4As are associated with CD8$^+$ T cell exhaustion in solid tumors, they might play different roles in acute leukemia, which requires further investigation.

Keywords: Leukemia, NR4A, T cell Exhaustion, TOX

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INTRODUCTION

During chronic infections and cancers, T cells become exhausted, where they overexpress immune checkpoint receptors and show reduced proliferation, effector cytokine production, and cytotoxic activity, leading to the failure of cancer elimination. Exhausted T cells are developed under the control of transcription factors, epigenetic factors, and metabolic enzymes (1).

Thymocyte selection-associated high mobility group box protein (TOX) and members of the nuclear receptor 4A (NR4A), including NR4A1, NR4A2, and NR4A3 (2) are known as essential factors for epigenetic alterations, changes in gene expression content, initiation and progression of T cell exhaustion (3, 4). Expression of *TOX* is abundant in exhausted T cells during chronic infections (5), where it plays an important role in the expression of immune-checkpoint receptors and reduced cytokine production. Expression of *TOX* depends on the calcineurin/nuclear factor of the activated T cells (NFAT) signaling pathway. Expression of the three members of the *NR4A* (*NR4A1-3*) also increases in exhausted T cells, while their absence prevents the development of T cell exhaustion. Expression of this group of transcription factors also depends on NFAT (4). The role of TOX and NR4A transcription factors in T cell exhaustion has been investigated in solid tumors, including breast and lung cancer (6).

Considering the important role of TOX and the NR4A family in inducing T cell exhaustion and their dependence on the calcineurin/NFAT signaling pathway, our study sought to evaluate the expression of these factors in $CD8^+$ T cell exhaustion in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML).

MATERIALS AND METHODS

Patients and Controls

The number of samples was determined in accordance with earlier research (1, 7, 8). Twenty-one patients diagnosed with ALL and 6 patients diagnosed with AML were recruited from the clinics and hematology/oncology wards at Imam Khomeini Hospital and Bou-Ali Sina Hospital, both of which are affiliated with Mazandaran University of Medical Sciences, Sari, Iran. The diagnosis of the disease was conducted through a comprehensive assessment, including clinical and paraclinical evaluations, based on the criteria established by the World Health Organization (WHO) (9) Demographic, clinical, and paraclinical data of the patient cohort were collected through the administration of a structured questionnaire. Additionally, a control group comprising 20 healthy volunteers was incorporated into the study. Patients suffering from chronic viral diseases, including human immunodeficiency virus (HIV) and viral hepatitis, a history of malignancies other than AML and ALL, autoimmune diseases, or taking immunosuppressive drugs, were not included in the study. About 8-10 mL of blood was collected from each participant in the study.

The study protocol was approved by the Ethical Committee of Mazandaran University of Medical Sciences. All patients and control subjects provided their signed informed consent form.

Isolation of $CD8^+$ T Cells by Magnetic-activated Cell Separation (MACS)

Peripheral blood mononuclear cells (PBMCs) were isolated from collected blood samples, using density gradient centrifugation on Lymphosep (Biosera, Nuaille, France). The cell viability was assessed using trypan blue staining. Next, the isolation of $CD8^+$ T cells from PBMCs was carried out using CD8 microbeads (Miltenyi Biotec, Germany). In order to evaluate the purity of isolated $CD8^+$ T cells, they were stained with fluorescent anti-CD8-FITC and anti-CD3-PE (Biolegend, USA). Subsequently, flow cytometry analysis was conducted, showing a purity of more than 99% (Fig. 1).

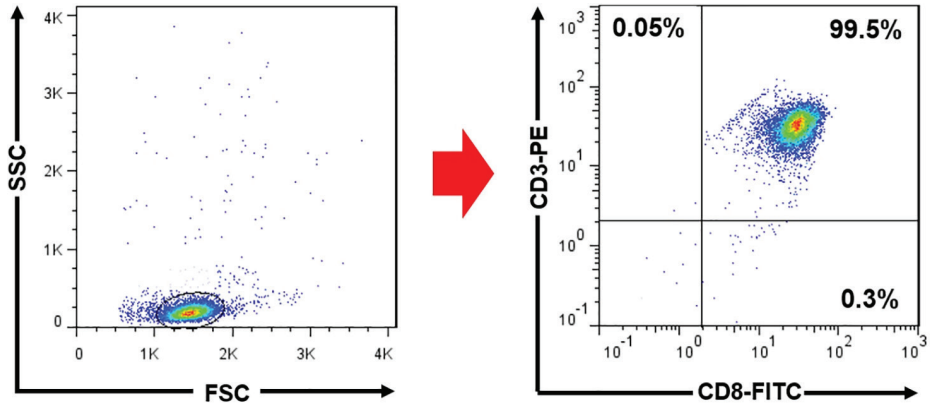

Fig. 1: Purity of MACS-separated CD8⁺ T cells. A two-color flow cytometry was performed on CD8⁺ T cells separated from the MACS column, using anti-CD3-PE and anti-CD8-FITC antibodies. Here is a dot plot chart of a sample that shows a purity of more than 99%. MACS: Magnetic-activated cell separation; PE: Phycoerythrin; FITC: Fluorescein isothiocyanate

Quantitative Reverse Transcriptase Polymerase Chain Reaction

The extraction of total RNA from CD8⁺ T cells was performed using the FavorPrep Blood/Cultured Cell Total RNA Mini Kit (Favorgen, Taiwan), according to the manufacturer's instructions. Next, the quantity of isolated RNA was confirmed, using nano-spectrophotometry. The quality of the isolated RNA was assessed through agarose gel electrophoresis. The synthesis of complementary DNA (cDNA) was conducted with the Yekta Tajhiz cDNA synthesis kit (Yekta Tajhiz, Tehran, Iran). Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) was carried out using the Real Q Plus 2x Master Mix (High Rox, Ampliqon, Denmark) with specific primers for *TOX*, *NR4A1*, *NR4A2*, and *NR4A3*, as well as *ACTB*, as a housekeeping gene (Table 1). The qRT-PCR was performed on an ABI Step One plus Real-Time system (Thermo Scientific). PCR was performed using 1 µL of cDNA, 10 µL of Master Mix, 10 pmol of each forward and reverse primer, and 7 µL of PCR-grade water. Samples were initially denatured at 95°C and amplified using 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and extension at 72°C for 30 seconds. Relative expression levels of *TOX*, *NR4A1*, *NR4A2*, and *NR4A3* were evaluated using the $2^{-\Delta\Delta Ct}$ method, after normalization to *ACTB* (10).

Statistical Analysis

Statistical analyses were carried out using SPSS 18 software. The results are presented as

Table 1: Primers used for qRT-PCR.

Gene	Primers (5'-3')*	Product size (bps)
TOX	F: TTC ACC ATC CAG TTC AGT R: ATA ACG CAT AGG CAG ACA	167
NR4A1	F: CTG GCG TAC AGG TCT AAG R: GTC GGT GAT GAG GAC AAG	192
NR4A2	F: TAT TCC AGG TTC CAG GCG AA R: GCT AAT CGA AGG ACA AAC AG	206
NR4A3	F: GGT AAA AGA AGT TGT CCG TA R: TGA GAA GGT TCC TGT TGT	93
ACTB	F: CCT TCC TGG GCA TGG AGTC CT R: TGG GTG CCA GGG CAG TGA T	174

*F: Forward primer; R: Reverse primer; TOX: Thymocyte selection-associated high mobility group box; NR4A: Nuclear receptor 4A; ACTB: β-actin

mean±SEM. The normality of the distribution of the examined parameters was assessed using the Kolmogorov-Smirnov (K-S) test. Based on the type of distribution and the quantity or quality of the variables, either parametric statistical tests (such as T test and analysis of variance) or non-parametric tests (such as Variance analysis and Kruskal Wallis) were employed. A significance level of $p<0.05$ was considered as the threshold for statistical significance.

RESULTS

Study Population

In this investigation, the sample population consisted of 21 ALL patients, among whom 8 were male and 13 were female, with a mean age of 15.94 years. Additionally, 6 AML patients were included, comprising 5 males and 1 female, with a mean age of 45.66 years. The research also encompassed a cohort of 20 control subjects, comprising 9 males and 11 females, with a mean age of 22.5 years. The main clinical and paraclinical characteristics of patients are summarized in Table 2.

TOX Expression in Patients and Controls

To evaluate *TOX* expression in $CD8^+$ T cells, we first isolated these cells from the blood of all study subjects using MACS, and after RNA extraction and cDNA synthesis, we evaluated the expression of this gene by qRT-PCR. The results showed no significant difference in the expression of *TOX* in ALL and AML groups compared with the control group ($p=0.9999$ and $p=0.7420$, respectively, Fig. 2).

Table 2: Major clinical and laboratory characteristics of study patients.

No.	Age (y)	Sex	WBC×10^3/mm^3	Lymphocyte (%)	PLT×10^3/mm^3	Hb (g/dL)	Leukemia subgroups
1	19	F	15.5	92.3	55	7	Pre B-ALL
2	18	M	7.5	57.4	165	9.5	AML–M1/M2
3	2	F	1.95	24.3	26	8.5	Pre B-ALL
4	2	F	2.79	79.9	308	8.9	Pre B-ALL
5	1	F	131.6	82.1	19	4.7	Pre B-ALL
6	49	M	12.0	21.0	70	12.4	Pre B-ALL
7	48	M	202.3	38.7	32	7.4	Pre B-ALL
8	69	F	8.9	90.8	76	7	Pre B-ALL
9	65	M	124.9	55.2	44	8.4	AML-M4
10	10	M	3.3	60.8	138	8	Pre B-ALL
11	14	F	10.72	49.1	34	12.5	Pre-B-ALL
12	51	F	31.0	87.1	19	9.5	Pre B-ALL
13	3	F	20.12	45.6	10	5.0	Pre B-ALL
14	4	F	5.7	58.1	120	7.9	Pre B-ALL
15	65	M	22.0	89.8	117	11.3	AML-M3
16	3	F	39.27	65.0	84	9.0	Pre B-ALL
17	88	M	82.8	64.0	33	7.2	AML-M1/M2
18	15	F	11.66	42.0	24	8.6	AML-M3
19	0.5	F	212.62	39.0	70	4.92	Pre B-ALL
20	3	M	11.19	60.0	42	9.4	Pre B-ALL
21	3	M	129.0	82.8	29	5.5	Pre B-ALL
22	2.5	M	80.0	62.0	87	7.9	Pre B-ALL
23	3	F	7.2	51.8	320	10.9	Pre B-ALL
24	5	M	49.7	32.0	208	3.2	Pre B-ALL
25	3	F	95.6	82.3	29	5.2	Pre B-ALL
26	8	M	8.4	72.3	157	9.6	Pre B-ALL
27	23	M	7.7	58.5	10	5.7	AML-M0/M1

M: Male; F: Female; WBC: White blood cell count; PLT: Platelet count; Hb: Hemoglobin; ALL: Acute lymphoblastic leukemia; AML, Acute myeloid leukemia.

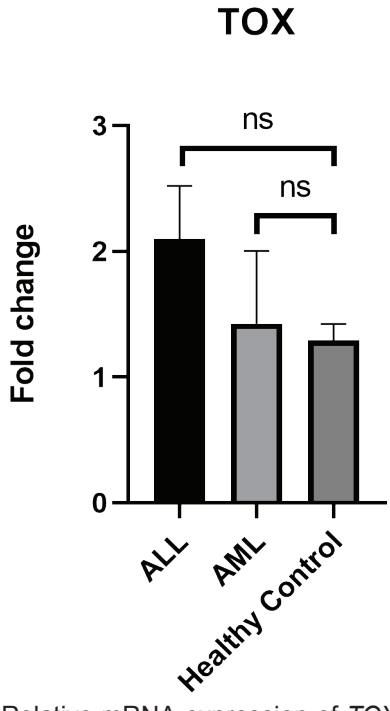

Fig. 2: Relative mRNA expression of *TOX* in ALL and AML patients and the controls. $CD8^+$ T cells were isolated from the peripheral blood of all study subjects using MACS. mRNA expression of *TOX* was then measured using qRT-PCR. *TOX* mRNA expression was first normalized to that of *ACTB* and then expressed as a fold change as the average expression level of the ALL or AML samples divided by that of the control samples. Vertical bars represent mean±SEM. *p<0.05; **p<0.01; ***p<0.001. TOX: Thymocyte selection-associated high mobility group box protein; ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; MACS: Magnetic-activated cell separation; qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction; ACTB: Actin beta

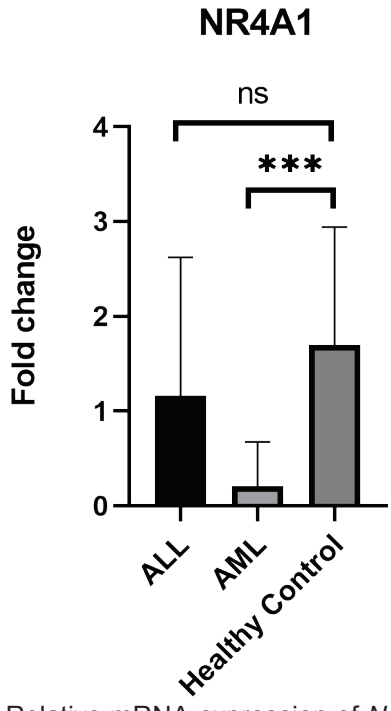

Fig. 3: Relative mRNA expression of *NR4A1* in ALL and AML patients and the controls. $CD8^+$ T cells were isolated from the peripheral blood of all study subjects using MACS. mRNA expression of *NR4A1* was then measured using qRT-PCR. mRNA expression was first normalized to that of *ACTB* and then expressed as a fold change as the average expression level of the ALL or AML samples divided by that of the control samples. Vertical bars represent mean±SEM. *p<0.05; **p<0.01; ***p<0.001. NR4A1: nuclear receptor 4A1; ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; MACS: Magnetic-activated cell separation; qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction; ACTB: Actin beta

Expression of NR4A1 in Patients and Controls

We also measured the relative mRNA expression of *NR4A1* in $CD8^+$ T cells from both ALL and AML patients, as well as the control group. Based on the qRT-PCR results, *NR4A1* expression in AML patients was markedly lower compared with the control group (p=0.0006), while not significantly different in ALL patients compared with the control group (p=0.0782, Fig. 3).

NR4A2 and NR4A3 Expression in Patients and Controls

In addition, we evaluated the relative mRNA expression of *NR4A2* and *NR4A3* in $CD8^+$ T cells from patients diagnosed with ALL and AML and the control group. Based on the results of qRT-PCR, the expression of the *NR4A2* gene was significantly lower in both ALL and AML groups than in the control one (p=0.0019 and p=0.0049, respectively, Fig. 4). Also, the expression of the *NR4A3* gene was significantly lower in both ALL and AML groups than in the control one (p=0.0055 and p=0.0005, respectively, Fig. 5).

DISCUSSION

In the current study, we assessed the mRNA expression of four T cell exhaustion-related

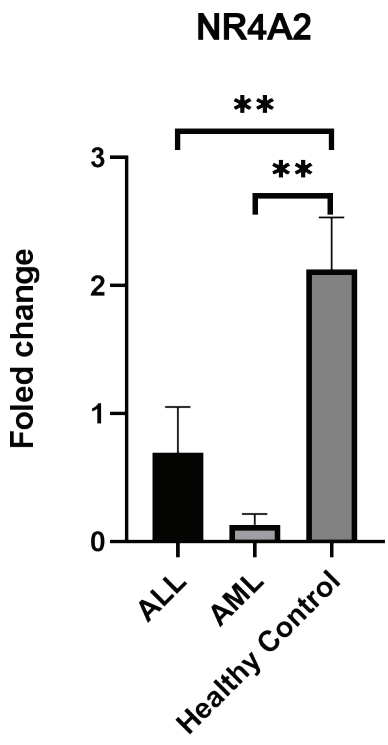

Fig. 4. Relative mRNA expression of *NR4A2* in ALL and AML patients and the controls. $CD8^+$ T cells were isolated from the peripheral blood of all study subjects using MACS. mRNA expression of *NR4A2* was then measured using qRT-PCR. *NR4A2* mRNA expression was first normalized to that of *ACTB* and then expressed as a fold change as the average expression level of the ALL or AML samples divided by that of the control samples. Vertical bars represent mean±SEM. **p*<0.05; ***p*<0.01; ****p*< 0.001. NR4A2: nuclear receptor 4A2; ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; MACS: Magnetic-activated cell separation; qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction; ACTB: Actin beta

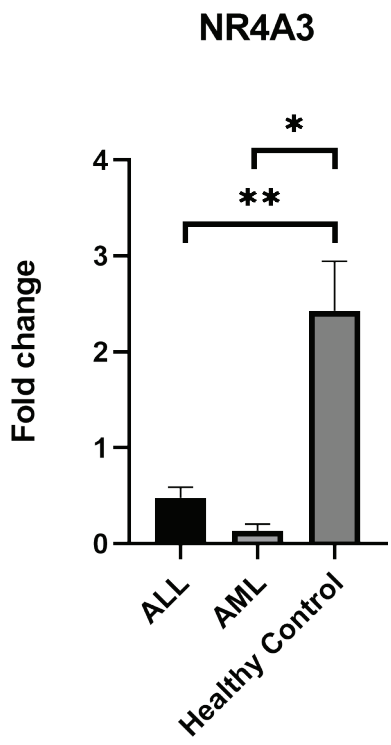

Fig. 5. Relative mRNA expression of *NR4A3* in ALL and AML patients and the controls. $CD8^+$ T cells were isolated from the peripheral blood of all study subjects using MACS. mRNA expression of *NR4A3* was then measured using qRT-PCR. *NR4A3* mRNA expression was first normalized to that of *ACTB* and then expressed as a fold change as the average expression level of the ALL or AML samples divided by that of the control samples. Vertical bars represent mean±SEM. **p*<0.05; ***p*<0.01; ****p*< 0.001. NR4A3: nuclear receptor 4A3; ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; MACS: Magnetic-activated cell separation; qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction; ACTB: Actin beta

transcription factors, *TOX* and *NR4A1-3*, in $CD8^+$ T cells in ALL and AML. The role of T cell exhaustion in the impairment of anti-tumor immune responses has been proved in several cancers (10).

Exhausted T cells are defined as non-effector cells; however, over the past five years, it has been shown that exhausted T cells could be divided into different stages, in some of which, they retain some levels of effector functions (11). Epigenetic analysis of exhausted T cells and comparison with effector and memory T cells revealed a huge difference in the active and available chromatin regions for transcription (12, 13). TOX and NR4As are known as essential factors for causing epigenetic changes, changing the content of gene expression, initiating, and promoting the process of T cell exhaustion (3, 4). Calcineurin is a calcium-activated serine/threonine phosphatase whose activation is necessary for positive selection of T cells in the thymus (14). Activated calcineurin results in nuclear translocation of transcriptional regulators, NFATs, by binding and dephosphorylating them in the cytoplasm. Expression of both *TOX* and *NR4As* depends on calcineurin signaling and NFAT (4). Also, blockade of NFAT reduces the expression and eliminates the effect of these factors (4).

The role of TOX and NR4As in T cell exhaustion in chronic viral infections and cancers has previously been investigated in several studies (5, 6), but few studies addressed the role of TOX in hematopoietic malignancies. In human solid tumors, it has been shown that the expression of all three members of the *NR4A* family increases in exhausted T cells. Moreover, animal studies have shown that gene knockdown of any of these factors is associated with an improved immune response against tumors or infections (4). In AML, a significantly higher percentage of TOX^+CD3^+, $CD8^+$, and $CD4^+$ T cells compared with the healthy individuals was reported (15). Another study reported an overexpression of *TOX* in the majority of T cell ALL and that TOX could inhibit repairing DNA damage and genomic instability and thus could play a role as an oncogenic factor (16). In our study, however, the expression of *TOX* in $CD8^+$ T cells from patients with ALL and AML did not show a statistically significant difference compared with those from the control group.

In our study, the expression of *NR4A2* and *NR4A3* was significantly lower in $CD8^+$ T cells from both AML and ALL patients compared with the control group. In solid tumors, it has been demonstrated that NR4As are involved in T cell exhaustion, as they are highly expressed in exhausted T cells compared with the pre-exhausted stage in both mice and humans (4). In hematopoietic malignancies, however, the role of NR4As in exhausted T cells has not been studied. In a study by Boudreaux et al., it was demonstrated that NR4As, particularly NR4A1 and NR4A3, function as tumor suppressors in the AML (17). In another investigation led by Wenzl et al., it was found that NR4A3 and NR4A1 serve as tumor suppressors in AML, and the simultaneous deletion of both nuclear receptors resulted in rapid development of AML in mice (18).

Over the past five years, it has been demonstrated that exhausted $CD8^+$ T cells exhibit heterogeneous phenotypes, from a progenitor to terminally-exhausted phenotype (11) Progenitor-exhausted $CD8^+$ T cells retain some levels of effector functions, thus to some extent, can control the progression of the tumor. They also respond to anti-PD-1 therapy (11). *TOX* and *NR4A*s have been suggested to be highly expressed in terminally-exhausted $CD8^+$ T cells rather than progenitor-exhausted $CD8^+$ T cells (19). We thus hypothesized that in acute leukemia, progenitor-exhausted $CD8^+$ T cells are more frequent than terminally-exhausted $CD8^+$ T cells, and thus, they do not express high levels of *TOX* and *NR4A*s.

In conclusion, the findings from this investigation reveal that although TOX and NR4As are associated with $CD8^+$ T cell exhaustion in solid tumors, they might play different roles in acute leukemia which requires further investigations. A constraint inherent to our investigation resided in the restricted enrollment of merely six AML patients. Consequently, the outcomes may not faithfully depict the precise gene expression profile characteristic of AML. To garner more elucidative data, it is imperative to undertake more expansive studies encompassing a larger cohort of AML samples.

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AUTHORS' CONTRIBUTION

Authors contributed to the study as coming: MT and HA designed and conducted the research. MM and AN carried out the assays. MM and BN contributed to data collection and analysis. HK, MN, MA, and RS collected and prepared the samples. RV assisted in the q-PCR assay and data analysis. MM, HA,

and MT drafted, revised, and finalized the manuscript. All authors read and approved the final version.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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