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CD8$^+$ T Cells in Acute Lymphoblastic Leukemia Show a Progenitor-exhausted Phenotype

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ABSTRACT

Exhausted T cells are phenotypically and functionally heterogeneous, from progenitor- to terminally-exhausted T cells. We evaluated gene expression profile of CD8$^+$ T cells in acute leukemia to characterize the phenotype of exhausted T cells.

Blood samples were collected from acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients prior to treatment and from control subjects. Additionally, samples were obtained from ALL patients after induction therapy. *TCF7, NFATc1, IRF4,* and *BATF* gene expression was then evaluated in isolated CD8$^+$ T cells.

CD8$^+$ T cells from ALL patients showed higher expression of *TCF7* and *NFATc1* compared to the control group. The two study groups did not have a significant difference in the expression of *BATF* and *IRF4*. When compared to the control group, CD8$^+$ T cells of AML patients showed an elevated expression level of *NAFTc1* and *IRF4*. Significant differences were not found between the two study groups in AML when it came to the expression of *BATF* and *TCF7*.

To our findings, the majority of CD8$^+$ T cells found in ALL patients consist of progenitor-exhausted T cells.

Keywords: SFA-2; Exhaustion; LSIRF; Leukemia; NFAT2; NFATc1; TCF-1

INTRODUCTION

Acute leukemia is characterized by improper differentiation and expansion of malignant hematopoietic progenitors. Acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) are two kinds of acute leukemia.[1-3] ALL, the most prevalent childhood cancer in children, originates from B- and T-lymphoid progenitors. The prevalence of ALL is fast rising, and it is estimated to affect 2 to 5 in 100 000 individuals annually, usually affecting children under the age of 6.[4] In adults, the most prevalent acute leukemia is AML, accounting for approximately 80% of acute leukemia cases.[5,6]

Chemotherapy, radiation therapy, and hematopoietic

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stem cell transplantation (HSCT) are current treatments for leukemia. However, they may cause adverse effects and are not applicable for all patients depending on their clinical condition.[7] Moreover, ongoing research is essential to develop more effective and affordable treatment options. Therefore, cancer immunotherapeutic strategies have gained importance nowadays. Like most cancers, ALL and AML are associated with T-cell exhaustion.[8,9] Exhausted T cells (TEX) display some characteristics, such as modified expression of some transcription factors, incorrect immune synapse construction, and boosted expression of immune-checkpoint receptors (ICRs), such as CTLA-4, PD-1, and Tim-3, which interact with their ligands and lead to weakness in proliferation, cytokine creation, and associated with a reduction in the cytotoxic activity of T cells. Although current ICR blockade therapy has shown considerable efficacy in several cancers, many patients fail to respond properly to these treatments, and certain tumor types remain largely resistant.

This is likely because our current understanding of ICR-mediated inhibitory pathways and TEX subsets remains incomplete, hindering the development of a comprehensive treatment approach. Studies have shown that dual-blockade of PD-1 together with TIGIT or TIM-3 fails to restore TEX functionality and has no effect on the characteristics of $CD8^+$ T cells.[10-13] Therefore, additional research in this field is essential to reach a reliable and effective therapeutic approach.

T-cell receptor (TCR)-responsive transcription factors, such as nuclear factor of activated T cells, cytoplasmic component 1 (*NFATc1*), interferon regulatory factor 4 (*IRF4*), and basic leucine zipper transcriptional factor ATF-like (*BATF*), are accountable for the differentiation of T cells.[14] *NFATc1* is a key member of the NFAT family and serves as an essential transcription factor for immune regulation. Following T-cell activation, *NFATc1* promotes initial PD-1 upregulation, suggesting the contribution of *NFATc1* in T-cell exhaustion.[15,16] *NFATc1*, in turn, regulates *BATF* and *IRF4* expression.[17] *BATF* is a bZIP transcription factor and plays a critical role in the control of differentiation and function of T cells. *IRF4* is a participant in the interferon regulatory family of transcription factors. Although previous studies have revealed that lack of *BATF, NFATc1*, and *IRF4* contributes to T-cell dysfunction, enhanced expression of *BATF* and *IRF4* mediate T-cell exhaustion in chronic infections and certain types of malignancies.[18-21] Transcription factor 7 (*TCF7*, also called *TCF-1*) is another transcription factor related to *NFATc1* pathway, which is responsible for T-cell self-renewal. Interestingly, studies have shown that *TCF7* is critical for the long-term preservation of T-cell responses, and that tumors with a higher frequency of $TCF7^+$ T cells showed better clinical outcomes.[22] Accordingly, activation of *NFATc1*, *BATF*, and *IRF4* represses *TCF7* expression in TEX.[14] Furthermore, *TCF7* plays multiple roles in the differentiation and activity of helper and regulatory T cells, as well as in regulating chromatin structure and accessibility. The existence of such diverse and unique functions is highly intriguing.[23]

The role of the *NFATc1* downstream pathway and *TCF7* in acute leukemia has not been studied. Therefore, this work was designed to assess the mRNA expression levels of these transcription factors in $CD8^+$ T cells from patients with acute leukemia, providing further insight into this pathway. Ultimately, determining the gene expression levels of our target genes can greatly contribute to our understanding of progenitor-TEX and terminally-TEX cells in patients with acute leukemias.

MATERIALS AND METHODS

Sample Collection

Based on the sample size used in comparable studies,[24-26] we enrolled 36 newly diagnosed ALL and 8 newly diagnosed AML patients who attended Imam Khomeini Hospital and Bou-Ali Sina Hospital (Sari, Iran). The World Health Organization (WHO) criteria were used for diagnosis.[27] Patients did not receive any prior treatments. To isolate acute leukemia as the sole pathological condition affecting T-cell and immune system function, we excluded participants with human immunodeficiency virus, and hepatitis B and C virus infections recognized as viral conditions linked to T-cell exhaustion,[19,28] congenital or acquired immunodeficiencies, history of immunosuppressive medications, prior malignancies, or autoimmune disorders. Twenty-five healthy subjects were also enrolled in the study, as controls.

Based on the questionnaire they filled out, they had no history of hematological disorders, malignancies, chronic viral diseases, or any other conditions related to the immune system. According to the ethics committee rules of Mazandaran University of Medical Sciences (MAZUMS), all participants read and signed written informed consent documents prior the enrollment.

Heparinized peripheral blood samples were obtained from all study subjects, ranging from 8 to 10 mL. Also, after 1 to 2 months of induction therapy, blood samples were obtained from 8 ALL patients.[29]

CD8$^+$ T-Cell Isolation

In the early step of purification, density gradient centrifugation on Ficoll-Histopaque was utilized to isolate peripheral blood mononuclear cells (PBMCs) from all samples (Biosera, Nuaille, France). Purification of CD8$^+$ T cells from PBMCs was achieved by means of magnetic-activated cell sorting with CD8 microbeads (Miltenyi Biotec, Germany). anti-CD8-FITC and anti-CD3-PE were utilized to examine and verify the purity of the isolated CD8$^+$ T cells (Biolegend, San Diego, USA) and read by flow cytometry. Figure 1 shows that the purity of the isolated CD8$^+$ T cells was over 99% in all cases.

Flow Cytometric Analysis

The isolated CD8$^+$ T cells underwent flow cytometric analysis, using anti-CD3-PE (Clone OKT3, Biolegend, San Diego, USA) and anti-PD-1-PerCP/Cy5.5 (Clone EH12.2H7, Biolegend, San Diego, USA), along with isotype-matched control antibodies. The samples were then examined using a Partec PAS flow cytometer (Partec GmBH, Munster, Germany) with FlowMax software for analysis.

Evaluation of Gene Expression

Whole RNA was extracted from the segregated CD8$^+$ T-cells population (Favorgen, Taiwan). A nano-spectrophotometer and electrophoresis were used to verify the quantity and quality of isolated RNA. Following synthesis of complementary DNA (cDNA) (Yekta Tajhiz, Iran), quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted with Real Q Plus 2x Master Mix (High Rox, Ampliqon, Denmark) reagent on an ABI StepOnePlus Real-Time system (Thermo Scientific) with specific primers for *BATF*, *IRF4*, *NFATc1*, and *TCF7* (Supplementary, Table 1), as well as *ACTB* (also called as β-actin), as a housekeeping gene. The results were normalized with those of *ACTB*, and relative expression levels of the subjected genes were determined using the $2^{-\Delta\Delta Ct}$ method.[30]

Statistical Analysis

Data assessments were carried out using the GraphPad Prism 6 (San Diego, CA, USA) and SPSS16 (North Castle, NY, USA) software. Results are presented as mean ± SEM. The nonparametric Mann-Whitney *U* test was employed to determine the mean difference between the groups, with statistical significance defined as $p<0.05$.

Study Participants

A total of 36 ALL patients (19 males and 17 females; mean age, 10.95 years), 8 AML patients (5 males and 3 females; mean age, 36.87 years), and 25 control subjects (16 males and 9 females; mean age, 18.8 years) were enlisted in the analysis. Table 1 provides a summary of main clinical and laboratory features of patients.

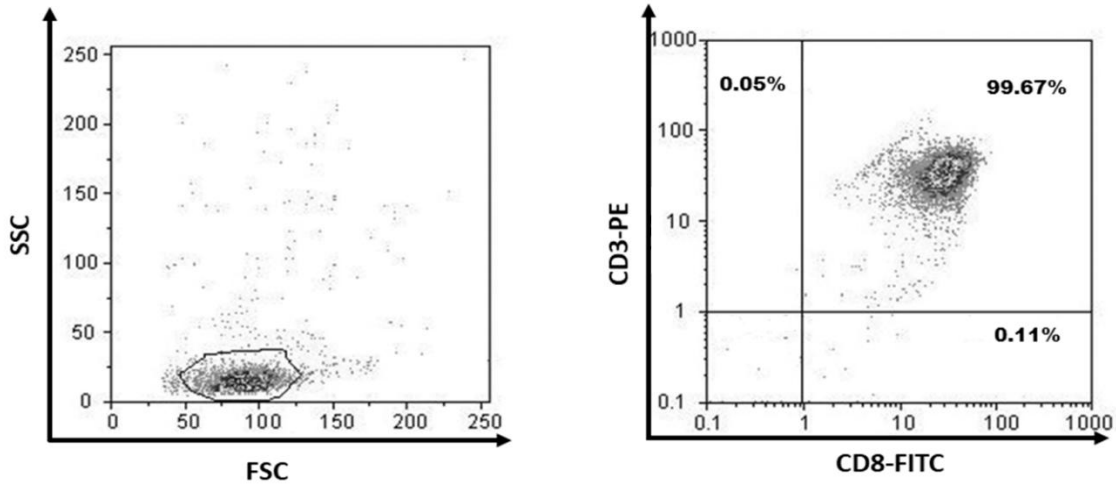

Figure 1. MACS separation of CD8$^+$ T cells and their purity. CD8$^+$ T cells were isolated from the MACS column by performing 2-color flow cytometry with anti-CD8-FITC and anti-CD3-PE antibodies. A sample with a purity of over 99% is shown on a dot plot chart. FITC: fluorescein isothiocyanate; MACS: magnetic-activated cell sorting; PE: phycoerythrin.

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Table 1. Major clinical and laboratory characteristics of patients

No.	Age, y	Sex	WBC, × 10^3/mm^3	Lym, %	PLT × 10^3/mm^3	Hb, g/dL	Leukemia subgroup
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	Pro B-ALL
12	51	F	31.0	87.1	19	9.5	Pro 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	Pro 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	58.8	10	5.7	AML-M0/M1
28	14	M	14	90.2	37	5.5	pre B-ALL
29	2	F	9.43	84.5	45	9.1	pre B-ALL
30	5	F	29.1	85.3	29	7.3	pre B-ALL
31	12	M	16.6	31	7	7.4	pre B-ALL
32	16	F	21.7	86	49	8	pre B-ALL
33	7	M	5.5	76	48	10.1	pre B-ALL
34	12	F	20.9	38.3	114	13.1	pre B-ALL
35	2	M	5.08	31.9	293	9.9	pre B-ALL
36	6	M	12.98	45.8	210	9.3	pre B-ALL
37	8	F	19.6	93.1	110	7	Pro B-ALL
38	1	M	6.10	45.4	273	10.1	pre B-ALL
39	0.25	F	5.29	14.7	334	9.1	Pro B-ALL
40	16	F	275	25	11 000	4.1	AML-M4
41	2	M	26.97	56	46	11.3	Pre B-ALL
42	4	F	19.4	26	19	8.6	AML-M4
43	14	M	131.32	80	67	6.7	Pre B-ALL
44	4	F	6.96	68.8	60	11.8	Pre B-ALL

ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; F: female; Hb: hemoglobin; Lym: lymphocyte percent in peripheral blood; M: male; PLT: platelet count; WBC: white blood cell count.

RESULTS

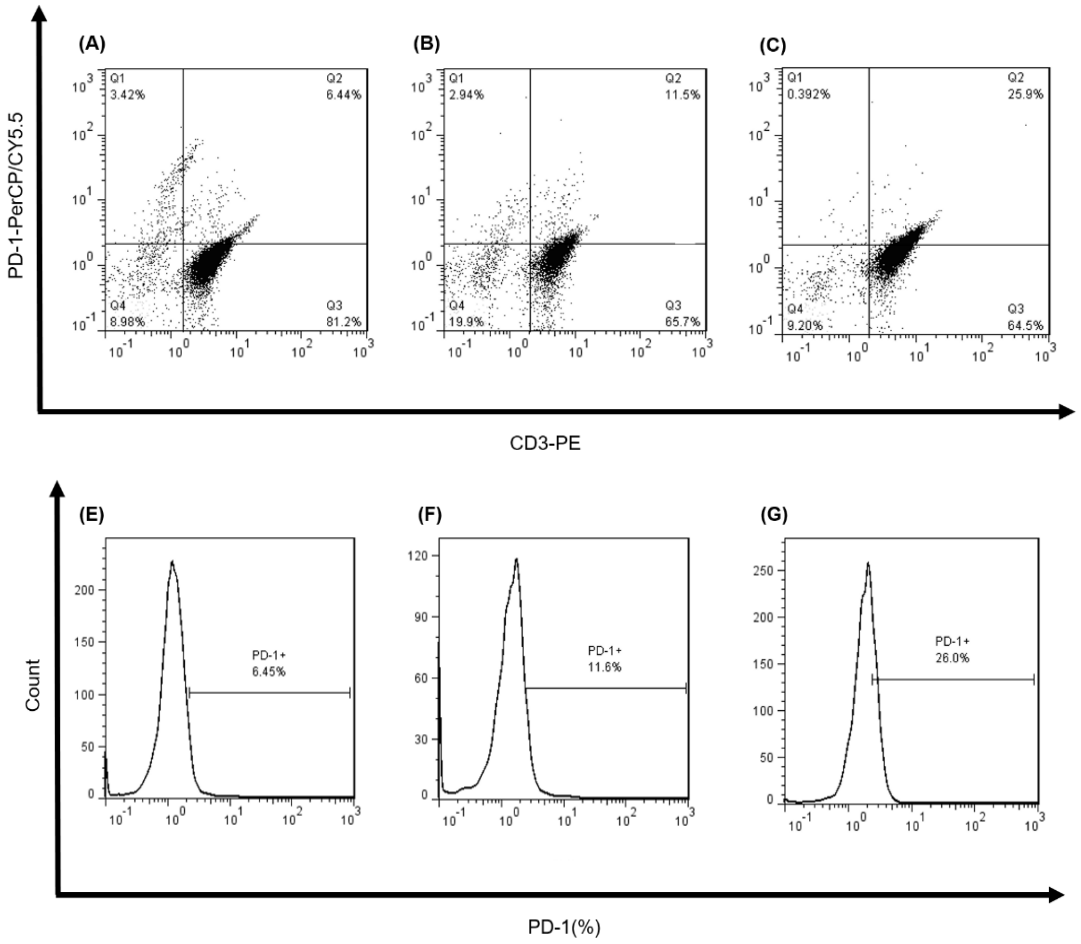
PD-1 Expression in CD8$^+$ T Cells

To verify the exhausted phenotype in CD8$^+$ T cells, flow cytometry was utilized to count the percentage of PD-1$^+$CD3$^+$ cells amongst Purified CD8$^+$ T cells (Figure 2). CD8$^+$ T cells from patients displayed a significant increase in PD-1 expression compared to control subjects (p=0.003, Figure 2H). We also assessed PD-1 expression after induction therapy, to determine the effect of induction therapy on T-cell exhaustion. The results showed no significant difference in PD-1 expression between baseline and post-induction therapy (p=0.375, Figure 2H), while PD-1 expression in treated patients was significantly elevated compared to control subjects (p=0.043, Figure 2H).

mRNA Expression of NFATc1-Related Factors

CD8$^+$ T cells from patients exhibited higher expression levels of *TCF7* and *NFATc1* compared to those from the control group (p=0.002 and p=0.037, respectively), The two groups did not display a significant difference in *BATF* and *IRF4* expressions (p=0.686 and p=0.524, respectively, Figure 3). In addition, to assess the effects of induction therapy on the expression of the *NFATc1*-related pathway, we also evaluated the expression of *NFATc1*, *BATF*, *IRF4*, and *TCF7* gene after 1 to 2 months of induction therapy, which showed no significant difference (p=0.261, p=0.512, p=0.092, and p=0.604, respectively, Figure 4).

In AML patients, expression levels of NAFTc1 and *IRF4* in CD8$^+$ T cells were higher than in those from the control subjects (p=0.026 and p=0.003, respectively). Besides, the two study groups did not display any remarkable differences in *BATF* and *TCF7* expressions (p>0.05 and p=0.078, respectively) (Figure 5).



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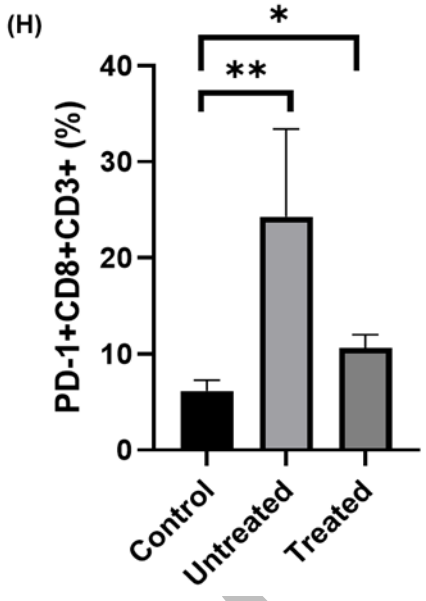

Figure 2. Frequency of exhausted CD8$^+$ T cells in ALL patients and controls. CD8$^+$ T cells from treated and untreated patients as well as control subjects were stained for CD3 and PD-1. Exhausted CD8$^+$ T cells were identified in the lymphoid region by CD3$^+$/PD-1$^+$ cells. Graphs show the percentage of PD-1$^+$CD8$^+$CD3$^+$ cells in control subjects (A and E), treated ALL patients (B and F), and untreated ALL patients (C and G). H. Comparison of PD-1$^+$CD8$^+$CD3$^+$ cells among control subjects (n=8), untreated ALL patients (n=8), and treated ALL patients (n=8). PD-1 expression in untreated and treated patients was significantly elevated compared with control subjects (p=0.003 and p=0.043, respectively). There is no significant difference in PD-1 expression between baseline and post-induction therapy (p=0.375). Vertical bars display the mean±standard error (SEM). *p<0.05; **p<0.01; ***p<0.001. ALL: acute lymphoblastic leukemia; PD-1: programmed cell death 1.

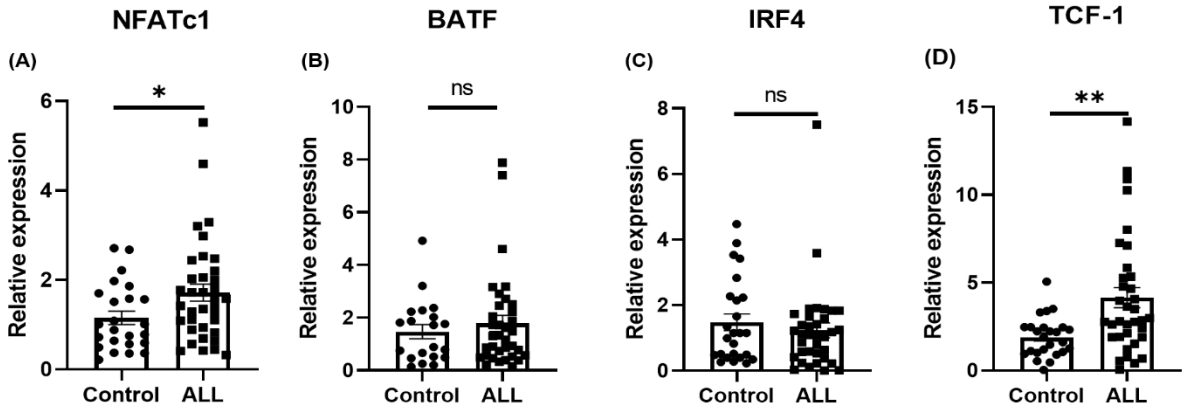

Figure 3. mRNA expression levels of *NFATc1*, *BATF*, *IRF4*, and *TCF7* in ALL patients (n=36) and control subjects (n=25), using qRT-PCR. Expression of each gene was initially normalized to that of *ACTB*, as a housekeeping gene, and subsequently expressed as the relative expression level of ALL samples divided by that of the control samples. *TCF7* and *NFATc1* significantly increase in patients (p=0.002 and p=0.037, respectively); *BATF* and *IRF4* expressions did not exhibit a significant difference (p=0.686 and p=0.524, respectively). Vertical bars display the mean±standard error (SEM). *p<0.05; **p<0.01; ***p<0.001. ALL: acute lymphoblastic leukemia; BATF: basic leucine zipper transcriptional factor ATF-like; IRF4: interferon regulatory factor 4; NFATc1: nuclear factor of activated T cells, cytoplasmic component 1; qRT-PCR: quantitative reverse transcription polymerase chain reaction; TCF7: transcription factor 7.

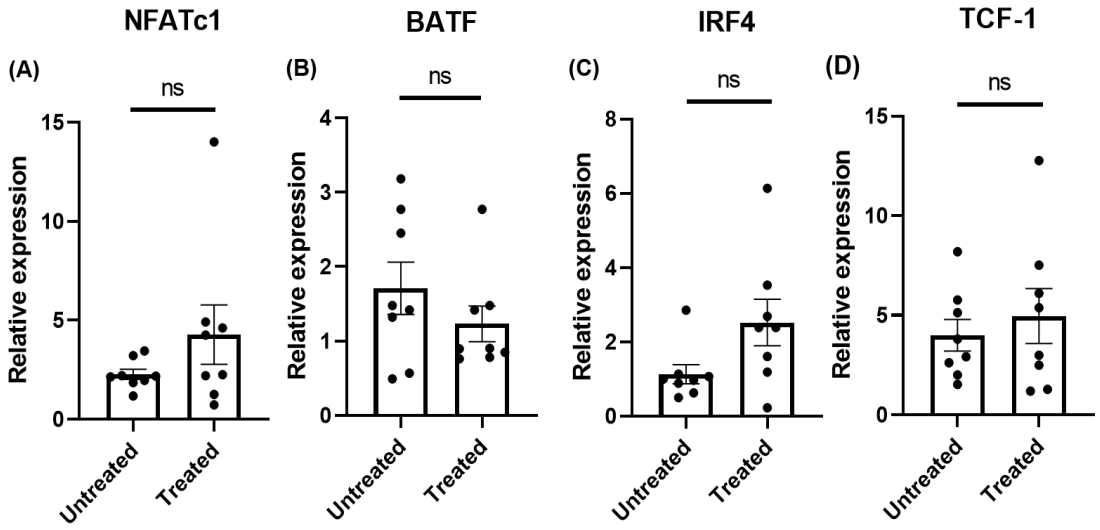

Figure 4. mRNA expression levels of *NFATc1*, *BATF*, *IRF4*, and *TCF7* in untreated (n=8) and treated ALL patients (n=8), using qRT-PCR. Expression of each gene was initially normalized to that of *ACTB*, as a housekeeping gene, and subsequently the relative expression levels of treated ALL and untreated patients were compared. There is no significant difference in *NFATc1*, *BATF*, *IRF4*, and *TCF7* expression after induction therapy (p=0.261, p=0.512, p=0.092, and p=0.604, respectively). Vertical bars display the mean±standard error (SEM). *p<0.05; **p<0.01; ***p<0.001. ALL: acute lymphoblastic leukemia; BATF: basic leucine zipper transcriptional factor ATF-like; IRF4: interferon regulatory factor 4; NFATc1: nuclear factor of activated T cells, cytoplasmic component 1; qRT-PCR: quantitative reverse transcription polymerase chain reaction; TCF7: transcription factor 7.

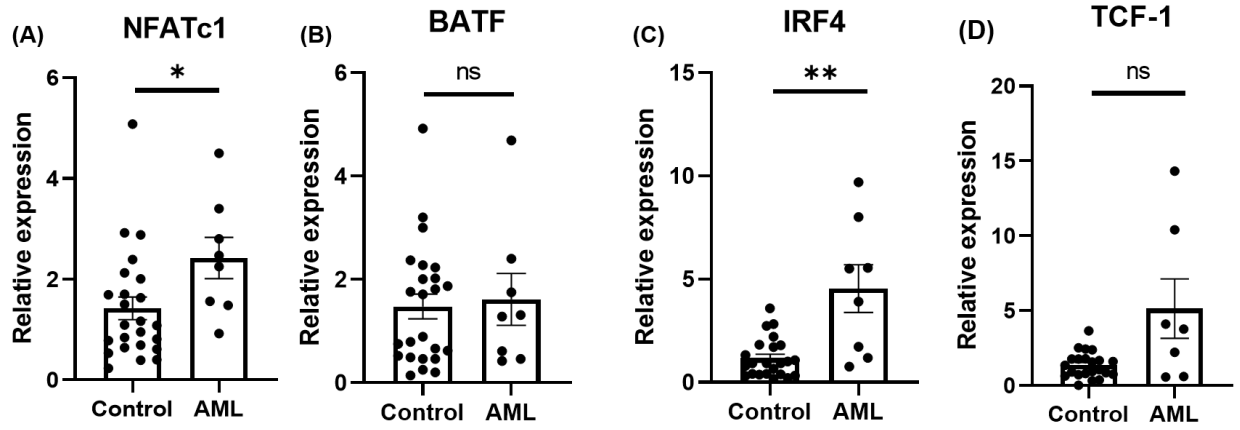

Figure 5. mRNA expression levels of *NFATc1*, *BATF*, *IRF4*, and *TCF7* in AML patients (n=8) and control subjects (n=25), using qRT-PCR. Expression of each gene was initially normalized to that of *ACTB*, as a housekeeping gene, and subsequently expressed as the relative expression level of AML samples divided by that of the control samples. Expression levels of *NFATc1* and *IRF4* in patients were higher than in those from the control subjects (p=0.026 and p=0.003, respectively). Study groups did not display any remarkable differences in *BATF* and *TCF7* expressions (p>0.05 and p=0.078, respectively). Vertical bars represent mean±SEM. *p<0.05; **p<0.01; ***p<0.001. AML: acute myeloid leukemia; BATF: basic leucine zipper transcriptional factor ATF-like; IRF4: interferon regulatory factor 4; NFATc1: nuclear factor of activated T cells, cytoplasmic component 1; qRT-PCR: quantitative reverse transcription polymerase chain reaction; SEM: standard error of the mean; TCF7: transcription factor 7.

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DISCUSSION

TCR-responsive transcription factors, including *NFATc1, IRF4*, and *BATF*, play a significant role in T-cells exhaustion, where they are significantly enriched in their binding site on promoters of PD-1 and other ICR genes, leading to their upregulation.[18] They also upregulate thymocyte selection-associated HMG box (*TOX*), a chief regulator of T-cell exhaustion, while downregulating *TCF7*. Exhausted $CD8^+$ T cells are heterogeneous and contain a subset of progenitor-TEX that differentiate into terminally-TEX. Unlike terminally-TEX, progenitor-TEX retains some level of effector functions and can survive without antigen stimulation enabling long-term persistence. These cells have been demonstrated to improve tumor control and respond better to PD-1 blockade therapy. *TCF7*, in turn, serves as a key regulator in the generation and preservation of progenitor-TEX, which are characterized as $TCF7^+PD\text{-}1^+$ T cells.[14,22,31-35]

In our work, we evaluated the gene expression of a network of TCR-dependent transcription factors, which is activated as a result of TCR stimulation, including *NFATc1, BATF*, and *IRF4*, in acute leukemia. As mentioned above, we demonstrated that *NFATc1* was significantly upregulated, while *BATF* and *IRF4* exhibited no remarkable differences in gene expression in ALL patients compared to control subjects. In AML patients, expression of both *NFATc1* and *IRF4* was significantly greater than in control subjects, whereas *BATF* expression showed no significant difference. Moreover, we evaluated the gene expression of *TCF7* to demonstrate whether *NFATc1* pathway could repress *TCF7*. The results showed that ALL patients exhibited $CD8^+$ T cells with a higher *TCF7* expression level compared to the control subjects. Concurrently, the expression of PD-1 in $CD8^+$ T cells from ALL patients was considerably greater than that of the control group. In our earlier study, we observed that *TOX* was not upregulated in $CD8^+$ T cells from ALL patients.[36] Notably, Shahbaz et al[37] illustrated that both Galectin-9 and VISTA were highly upregulated during viral infection and that the coexpression of Galectin-9 with PD-1 resulted in a terminally-TEX. It has been shown that T cells coexpressing Galectin-9 and PD-1 exhibit reduced proliferative capacity and cytokine production compared to those expressing one of these molecules. However, we previously demonstrated that neither Galectin-9 nor VISTA were upregulated in $CD8^+$ T cells from patients with B-ALL, with Galectin-9 levels showing a significant decrease.[37,38] Consequently, it can be suggested that in ALL, the majority of $TCF7^+CD8^+$ T cells are progenitor-exhausted $CD8^+$ T cells. This evidence is in line with prior studies that have shown that in mouse models of Philadelphia chromosome-positive B ALL, anti-PD-1 therapy alone had an improvement in median overall survival.[39]

Our findings in AML patients indicated that the gene expression of *TCF7* did not show a remarkable difference between the patients and the control individuals. There is not much research on the number and role of progenitor-TEX in AML. One study showed that AML patients who had a higher number of *TCF7*-expressing $CD8^+$ T cells before PD-1 blockade therapy, responded better to therapy, and had a greater recovery.[40] Another study showed augmented expression of *TOX* in $CD8^+$ T cells in AML.[41] Although our findings provide valuable insight, the limited number of AML patients in this study necessitates further investigations with a larger number of AML patients to achieve a more accurate interpretation.

We further assessed *NFATc1*, *BATF*, *IRF4*, *TCF7*, and *PD-1* expressions after 1 to 2 months of induction therapy in ALL patients for the completion of our study. We showed that *NFATc1*, *BATF*, *IRF4*, *TCF7*, and *PD-1* expressions were not significantly different after therapy, indicating that TEX phenotype might still remain in patients after induction therapy.

Moreover, previous studies have reported that prolonged TCR stimulation could lead to the formation of a transcription factor network, including *NFATc1*, *BATF*, and *IRF4*. These transcription factors collectively, rather than individually, bring about a self-reinforcing transcriptional loop that induces *TOX* expression while repressing *TCF7*,[17,18,33] ultimately leading to formation of terminally-TEX. In line with this, our previous study on ALL patients demonstrated that *TOX* was not upregulated in $CD8^+$ T cells.[36] Also, the present study showed that *BATF* and *IRF4* were not upregulated in $CD8^+$ T cells in ALL.

We hypothesized that insufficient function of any of transcription factors, including *NFATc1, BATF*, or *IRF4*, could disrupt the network. Alongside, Man et al recently reported that the transcription factor trio, *NFAT, BATF*, and *IRF4*, represses *TCF7* expression. It has been shown that TEX express high levels of the transcription factor *NFATc1*, which is required for the TCR-dependent induction of *IRF4* and *BATF*. Accordingly, inhibition of

NFATc1 activity resulted in strongly reduced *IRF4* protein levels and *BATF* expression. Consistent with these findings, the gene encoding *TCF7* was identified as a direct transcriptional target of *IRF4*. Furthermore, blocking *IRF4* expression was sufficient to restore *TCF7* expression, allowing the development of memory-like T cells, reducing expression of ICRs, and significantly restoring production of IFN-γ.[18]

One limitation of our study was that most of the patients were children under 18, as ALL is predominantly observed in pediatric population. However, the control group consisted of individuals aged 18 to 21 years, since research ethics regulations do not permit sampling from healthy individuals under 18 years of age.

Collectively, our results suggest that majority of $CD8^+$ T cells in ALL patients are progenitor-TEX, as they coexpress *TCF7* and PD-1. Given that progenitor-TEX have been shown to be activated more effectively against malignant cells,[14] PD-1/PD-L1 blockade seems to be effective in ALL, as it augments progenitor-TEX. On the other hand, based on our results and previous studies, blockade of *NFATc1, BATF*, and *IRF4*, either separately or simultaneously, is unlikely to be beneficial for ALL patients. More investigations and inquiries are necessary to comprehend the interaction between these transcription factors and the function of progenitor-TEX in malignancies, particularly in acute leukemia.

STATEMENT OF ETHICS

Written informed consent was obtained from all participants. The Ethics Committee of MAZUMS approved the procedure for conducting the study.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The datasets are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

The authors declare that no AI tools were employed in the creation of this article.

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