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Expression of immune checkpoint molecules in Iraqi acute myeloid leukemia patients

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Abstract:

BACKGROUND: Acute myeloid leukemia (AML) is a malignant disease of the bone marrow in which hematopoietic precursors are arrested in an early stage of development leading to production of abnormal cells. AML is the most common type of leukemia in adults. The most important advances can be achieved through immune checkpoint (IC) inhibitors, including cytotoxic T lymphocyte antigen 4 (CTLA-4), Programmed Death protein -1 (PD-1), and anti-programmed ligand 1 in cancer treatment over the past decade.

OBJECTIVES: The aims of the current study were to evaluate the expression of CD3, CD28, CD152, CD223, and CD279 markers in T cells by flow cytometry and the expression of PD 1, CTLA 4, and lymphocyte activation gene 3 (LAG 3) expression by real time PCR in AML patients.

MATERIALS AND METHODS: This is case control study carried out on 50 AML Iraqi patients, in addition to 50 apparently health person. This study was conducted at the National Center for Hematology, Department of Biology, Mustansiriyah University, and in Baghdad teaching hospital in Medical City, from January 2019 to June 2020. Moreover, the study aims to evaluate the expression of CD3, CD28, CD152, CD223, and CD279 markers in T cells by flow cytometry of AML patients and the expression of PD 1, CTLA 4, and lymphocyte activation gene 3 (LAG 3) expression by real time PCR in AML patients.

RESULTS: The cellular expression of almost CD markers did not show a relationship between gender and age. Most AML patients had high CD3 and CD28 expression in cellular expression of T cells. Although there were increasing gene expression of PD-1 and LAG-3 in T cells . The cellular expression of CD279 PD-1 was high , gene expression of CTLA-4 had slightly increased, and cellular expression of CD152 CTLA-4 not significant among healthy controls. In the present manuscript, there was an increase in the expression of PD-1 CD279 in the relapse and refectory patients than the complete and partial remission, while CD3, CD28, and CD223 LAG-3 did not show differences in the expression on T cells among AML stages. Finally, the immunophenotyping of 48 from 50 patients (96%) of the present study was CD3+CD28+CD152−CD279+CD223−.

CONCLUSION: Elevate the expression of PD 1, LAG 3 in almost all AML patients associated with the progression of the disease. 96% of AML patients' immunophenotyping was CD3+CD28+CD152−CD279+CD223−.

Keywords:

AML, cytotoxic T lymphocyte antigen 4, flow cytometry, lymphocyte activation gene-3, PD-1

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Introduction

It has been a dream in hemato-oncology to use the human beings' own immune system to eradicate cancer cell. A better understanding of the interaction between the immune system and tumors in recent years has given rise to new and powerful forms of immunotherapy. This therapeutic strategy was born from the understanding that tumors can circumvent the host immune system by usurping the pathways of the

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immune control point, such as the protein 4 (cytotoxic T lymphocyte antigen 4 [CTLA-4]) associated with cytotoxic T lymphocyte, and the pathways of programmed death 1 (PD-1).[1]

Now, it is better to understand the mechanisms that prevent the complete and receptive immune response to cancer cells. CTLA 4 is a protein associated with cytotoxic T lymphocytes, has a clear structural homology to CD28 in recent years used antibodies targeting CTLA-4 used as an agonist or antagonist. Secondary, stimulants.[2,3]

In 2013, cancer immunotherapy was the invention of the year. Happiness is primarily focused on the clinical performance of antibodies targeting CTLA-4 and PD-1 (protein 1 programmed for apoptosis) to modulate immune checkpoints (ICs).[4]

Eventually, T cell response includes signals from pro-inflammatory cytokines, especially interleukin (IL)-12, IL-21, and type 1 interferon (IFN-alpha/beta). 2 Blocking CTLA 4 and PD 1 with monoclonal antibodies is a pioneering technique in cancer treatment (a) because these molecules completely ignore cancer cells and rely solely on the immune system and (b) because it is not used to stimulate the immune system against some types of cancer but is used to neutralize inhibitory molecules that block positive T cell responses.[5]

Acute myeloid leukemia (AML) is a heterogeneous blood disease characterized primarily by impairment of myeloid differentiation and expansion of immature myeloid ancestors (blasts) in the bone marrow (BM) of patients. While statistically it is a relatively rare type of cancer (1.1% of all new cancers), according to the American Cancer Society's projections, in the United States, chronic myeloid leukemia (CML) will affect nearly 20,000 people in 2019.[6]

Central to this dilemma is the absence of clear evidence, in the form of pathogen-associated molecular patterns (PAMPs) that an immune response is warranted. To compound matters further, cancers frequently behave similar to wounds, attracting the attentions of the immune system but receiving a helping hand rather than the hostile response that would be more appropriate.[7]

Because tumors are self and are not typically associated with infectious agents (although there are some important exceptions to this), such cells lack PAMPs that are normally required to get robust immune responses off the ground. Thus, although the mutational processes associated with the development of cancer frequently generate neoantigens that, in principle, can elicit T cell responses, in practice, such responses are highly muted because of mechanisms that serve to prevent the emergence of autoimmunity. T cell activation involves dual signaling as described in the two-signal theory. The first signal is provided by the interaction of the T cell receptor (TCR) with an analogous antigen and the interaction of the major histocompatibility complex (MHC) with the antigen presenting cells (APCs). The costimulatory molecules, expressed on the APC surface, are responsible for a second signal known as the costimulatory signal. The lack of costimulation results in nonresponsive T cells, known as anergy.[8] As a consequence, well-meaning regulatory T (Treg) cell responses and other mechanisms that serve to limit the development of autoimmunity (such as CTLA-4- and PD-1-mediated downregulation of T cell responses) conspire to suppress the immune response against cancer. Moreover, tumors also actively manipulate the immune system to minimize immune responses that do emerge. Indeed, there is a growing body of evidence that tumors frequently recruit macrophages, neutrophils, as well as other innate immune cells, and "re-educate" such cells toward a wound-healing phenotype for the purpose of supporting tumor growth and survival.[9]

Another major impediment to the development of robust antitumor immune responses is the fact that tumors arise in a stepwise fashion, over long periods of time, which permits the selection of cells that are effectively invisible to the immune system. If they are not, such cells are weeded out by the immune system as the tumor develops.[10] Many tumors escape immune surveillance by downregulating positive costimulative molecules and upregulating coinhibitory signals. Blocking of the coinhibitory path way and activating the positive signals that lead to enhancing antitumor immunity.[11]

Cancers arise more frequently in the tissues that exhibit a high rate of mitosis probably because these cells are already dividing at a relatively high rate and the barriers to cell division are lower than in nondividing (i.e., postmitotic) tissues. Since dividing cells need to replicate their genomes, a process that can itself be a source of mutation because of errors made by DNA polymerase, such cells can be a source of genetic instability.[9] These soluble mediators can recruit neutrophils and macrophages, which in turn produce additional cytokines, growth factors, and other soluble factors that promote proliferation of the tumor as well as the growth of new blood vessels (angiogenesis) that are required for rapidly proliferating cells. Macrophage density correlates with a poor prognosis in approximately 80% of cancers, and there is now much evidence that tumors frequently "re-educate" macrophages through the provision of anti-inflammatory cytokines (such as IL-10 and tumor growth factor-β) that can generate an anti-inflammatory environment within the tumor. This can lead to the suppression of any T cell responses that

do emerge.[12] Thus, the recruitment of macrophages to tumors can generate an environment that conspires to help rather than fight the tumor. As if this was not already bad enough, there is increasing evidence that tumor-associated inflammatory cells, especially macrophages and neutrophils, can even promote the progression to malignancy and metastasis through the production of reactive oxygen and nitrogen species that can provoke DNA damage and thus generate additional mutations. Thus, tumors can manipulate cells of the immune system for their own ends, which further contributes to the difficulty of developing tumor immunity.[9,13]

Immune checkpoint inhibitors

IC inhibitors (ICIs), including CTLA-4, antiapoptotic cell death 1 (PD-1), and anti-PD ligand 1 (PD-L1), may be the most significant advances in treatment cancer over the past decade. Indicators of such factors continue to expand depending on malignant neoplasms and diseases, thereby changing many strategies of previous standards of treatment and giving patients a new hope.[14]

Cytotoxic T lymphocyte antigen 4 (CD152)

CTLA-4, also referred to as CD152, is one of the first negative regulators to be stimulated and directly compete with CD28 for CD80 and CD86 ligands. CTLA-4 (CD152) and CD28 are homologous receptors that are expressed by both CD4+ and CD8+ T cells and mediate conflicting roles in T cell activation. Both receptors have a pair of ligands that are expressed on the surface of APCs. CD28 interacts with relatively high affinity CD80 dimer and low affinity CD86 monomer, resulting in T cell costimulation along with TCR signaling.[15,16]

CTLA-4 is a receptor expressed on the surface of T cells that modify the cosignaling of CD28 by acting on activation ligands. CD80 and CD86 expressed on the surface of antigenic cells at an early stage of the immune response, thus attenuating the activation of T cells. CTLA-4 activation blocks the NF-µB signaling pathway, resulting in the inhibition of IL-2 production.[14] IL-2 is a powerful immunomodulatory cytokine that activates tumor cytotoxic T lymphocytes and natural killer (NK) cells; IL-2 has potential antileukemic effects.[17]

This was shown to upregulate CD80 and CD86 on AML blasts.[18-20] Such ligands may have the ability to suppress effector T cells by direct interaction with CTLA-4 on normal T cells. Blocking CTLA-4 in preclinical models leads to enhanced responses of T cells against AML. Furthermore, it has been shown that CTLA-4 is mainly expressed on the surface of AML blasts in patients at the time of diagnosis and in patients with a disease resistant to chemotherapy. CTLA 4 activity [Figure 1a] using the ligands CD80 and CD86 was able to induce leukemic cell death.[21 23]

Since CTLA-4 inhibits the CD28 pathway, which plays a role in assisting T cells in B cell responses, CTLA-4 deficiency is expected to increase CD28 activity and enhance humoral immunity. One possible explanation is that increased T cell activity can lead to invasion and destruction of the niche of the BM, thereby impairing the development of B cells.[24]

Programmed death-1 (CD279)

Blocking of the IC by programmed cell death inhibitors 1 (PD-1) and PD-1 ligand 1 (PD-L1) significantly improved clinical outcomes for a variety of solid tumors, although little is known about the function of immune checkpoint activators (ICAS) in blood cancer. Previous studies have shown that increased PD-1+ T cell counts are associated with poor outcomes in AML patients.[25]

Clinical studies using PD-1 inhibitors are underway in the treatment of high-risk AML relapse patients. However, the response rate varies widely from 22% to 72%,[26] which may be due to the heterogeneity of IC

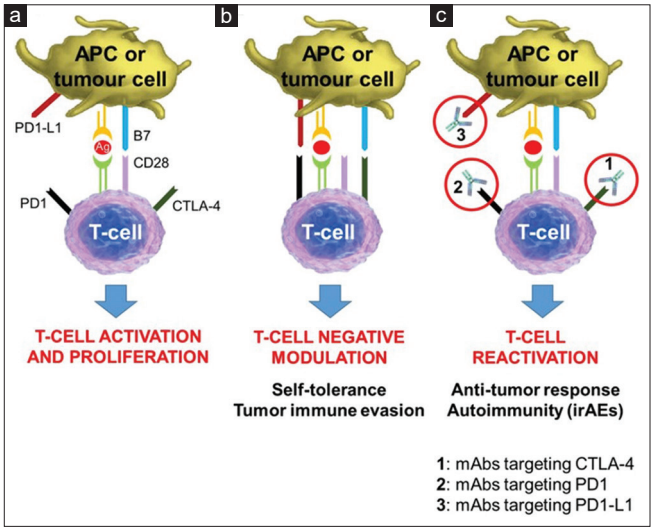

Figure 1: Mechanism of Action of Immune Checkpoint Inhibitors.Tumor antigens are presented to T-cells by antigen-presenting-cells (APCs) via the interaction of the major histocompatibility complex (MHC) and the T-cell receptors, representing the primary signal for activating T-cells. Another costimulatory signal involving interaction between B7 on APCs and CD28 on T-cells is needed to complete T-cell activation and expansion (Panel A). Several co-receptors act as negative modulators of immune response at different molecular checkpoints. The CTLA-4 is induced in T-cells at the time of their initial response to antigen. CTLA-4 is transported to the cell surface proportionally to the antigen stimulation; it binds to B7 with greater affinity than CD28, resulting in specific T-cell inactivation (Panel B). The PD-1/PD1-L1 pathway is not involved in initial T-cell activation. It regulates inflammatory responses in tissues sustained by effector T-cells. Activated T-cells up-regulate PD-1 and inflammatory signals in the tissue induce the expression of PD1-L1s, which downregulate the activity of T-cells, protecting normal tissues from collateral destruction; this mechanism is also exploited by tumor cells to evade the immune system response (Panel B). Monoclonal antibodies that block either CTLA-4 or PD1/PD1-L1 increase cytotoxic T-cell activity by expanding T-cell activation and proliferation (Panel C). The eventual T-cell reactivation is responsible for the both anti-tumor response and the immune-related adverse events associated with these drugs.(Corsello *et al.* 2013)

expression rate in addition to the clear dominant trends in the IC expression in different AML states.[27]

Programmed cell death protein 1 (PD 1, also known as PDCD1 and CD279) is also expressed during T cell activation and opposes positive signals via TCR and CD28 by engaging its bonds with ligand 1 of programmed cell death 1, and inhibitory signals are used to maintain stability of the immune system in different ways [figure 1 b,c]. PD-1 becomes the model for recognizing inhibitory receptor complex physiological functions. Signaling through the PD-1 pathway contributes to the regulation of initial T cell activation, fine-tuning of T cell fate and function, T cell tolerance, and return to immune homeostasis.[28] PD-1 is a negative costimulatory receptor on the activated T lymphocytes that counteract the activation signal provided by the TCR ligation. PD-1 can also be stimulated in the NK cells, B cells, and monocytes. The two ligands of PD-1 are PD-L1 and PD-L2. They have distinct cellular expression patterns. PD-L2 expression is largely restricted to antigen-containing cells, whereas PD-L1 is widely expressed in the tissues and may be IFN induced. Exposure to PD-L1 IFN is the main link for PD-1-mediated immune suppression.[29] By causing inhibitory signals, PD-1 contributes to T cell exhaustion, a state in which T cells lose their effector functions, upregulate various inhibitory receptors, and lose their ability to attain a quiescence status.[30]

The expression patterns of PD-1, PD-L1, and PD-L2 were further analyzed along with other significant ICs.[31] Subsequently, the authors observed that the expression of PD-1, PD-L1, or PD-L2 was positively correlated with the expression of the protein 4 correlated with cytotoxic T-lymphocyte (CTLA-4) and lymphocyte activation gene-3 (LAG-3).[32] Further, the results indicate increased in PD-1+ CD3+ cells.[33] The relapsed AML patients after autologous stem cell transplant had a higher level of PD-1/T cell immunoglobulin (Ig) and a mucin-domain containing-3 (TIM3)-positive T cells in their PB, and these T cells displayed signs of exhaustion in response to CD3/CD28 stimulation.[34]

The most remarkable finding was an increased number of PD-1+ CD3 cells, and the majority of these PD-1+ cells were CD8+ cells, whereas the number of PD-1+ CD4+ cells in the AML group was not increased, which may be associated with a cytotoxicity dysfunction of T cells in AML.[35]

However, AML progression studies of these inhibitory pathways are limited and often preclinical. In an AML mouse model, in which AML was induced by intravenous injection of C1498 (a murine leukemia cell line), PD-L1 elevation on leukemia cells was observed, and less progression in PD-1−/− mice as well as PD-L1 blockage was also achieved.[36] Another gene expression study in myelodysplastic syndrome (MDS) and AML patients shows inhibition of PD-L1 and PD-L2 in leukemic blasts, showing a role for this PD-1 pathway in AML pathogens.[37] Recent findings about the immunomodulatory role of gut microbiota shed light on new ways to improve immunotherapy for cancer.[38]

Lymphocyte activation gene-3 (CD223)

In an experiment designed to selectively isolate molecules expressed in an IL-2-dependent NK cell line, LAG-3 was initially discovered.[39] It is a protein found on the surface of activated T cells, Treg cells, NK cells, B cells, and dendritic plasmacytoid cells. LAG-3 signaling prevents the activation of T cells and improves the regulatory function of T cells.[40] Another primary IC receptor expressed on activated or exhausted T cells is LAG-3, which was first identified as a member of the novel eukaryotic LAG-1 protein family and postulated to mediate sphingolipid metabolism in 2003.

LAG-3, a superfamily member of Ig, is a type I transmembrane protein with four Ig-like, extracellular domains. Lag-3 expressed on the activated T cells, NK cells, or B cells and negatively regulates the homeostasis of these cells.[41] LAG-3 has been identified as a new-generation Immune checkpoint (IC) protein.[42] It plays numerous functions including inhibition of Th1 cell proliferation and reduced IL-2, IFN-α, and tumor necrosis factor in T cells.[43] Structural, LAG-3 (also known as CD223) is similar to CD4, but it has a higher affinity to MHC Class II molecules than CD4.[44]

LAG-3 is associated with the complex CD3/TCR which suppresses T cell activation.[45] LAG-3 is considered a receptor of a novel IC for stimulating antitumor T cells.[46]

LAG-3 helps tumor cells to avoid immune surveillance during tumor genesis and cancer progression. LAG-3 blockade alone seemed to have no effect, and the synergistic effect of adding PD-L1 blockade strengthened the response of CD8+ and CD4+ T cells.[47] Recent review articles suggest that LAG-3 may serve as a target for cancer immunotherapy because it negatively regulates T cell function and may mediate a state of exhaustion in combination with PD-1.[48] These markers include PD-1, CTLA-4, LAG-3, and TIM3-suppressing T cell activation.[49] Furthermore, LAG-3 and PD-1 work synergistically regulating the function of T cells.[50] The expression of LAG-3 can be assessed by flow cytometry (FCM) on the surface of T cells in the PB. Immunohistochemistry can be used to determine the expression of LAG-3 in the tissue samples.[51] Cancer cells escape the surveillance of the immune system by various mechanisms, including activation of these specific immune control pathways, which suppress immune responses to

antitumors.[52] Gene-3 activation of lymphocytes (LAG-3) and CTLA-4 are considered a significant inhibitory IC expressed on the T cells.[53] Negative regulators are very important for adaptive immune response and play a key role in maintaining peripheral tolerance.[54] Due to overstimulation of antigens induced by cancer cells, reactive T cells slowly reach a dysfunctional state, which is called T cell exhaustion. In this regard, the presence of IC receptors is a sign of "burnout."[55] There are several IC molecules that can further identify damaged T cells, such as LAG-3. These receptors increase with age and become the future of endogenous amplification therapy aims.[56] The use of second immunomodulatory monoclonal antibodies (also known as ICIs) to improve immune suppression screening can increase the antitumor response.[57] Compared with the current control group, all CTLA-4 and LAG-3 immunoassay molecules in AML patients are significantly upregulated, indicating that the expression levels of CTLA-4 and LAG-3 in AML patients are not affected by the patient's age or have CTLA-4 and four ways. CTLA-4 and LAG-3 play a clear and relevant role in AML because CTLA-4 and LAG-3 may be the predictive markers for AML patients.[58]

Materials and Methods

The study was conducted on 50 Iraqi patients with acute myeloid leukemia and 50 matched healthy looking subjects. The age range of the patients was 17–80 years. This study was done in the National Center of Hematology, Department of Biology at Al Mustansiriyah University, and Baghdad Medical City from January 2019 to June 2020. Each patient was subjected to physical examination done by a specialist, and illness–information related to this research was obtained.

In this research, the expression of immunophenotyping CD3, CD28, CD152, CD223, and CD279 was studied using fully fitted eight–color FCM desktop. CyFlow Cube has an optical idea that is modular. This enables distinct lasers to be used as sources of light. The CyFlow Cube enables a simple exchange of optical filters and mirrors to optimize the optics for any implementation.

The rates of expression of the genes PD-1, CTLA-4, and LAG-3 were measured using quantitative real-time (qRT)-PCR. qRT-PCR SYBR Green Assay was used to confirm the target gene expression. The primer sequences for the genes PD-1, CTLA-4, and LAG-3 were prepared using synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at – 23°C; the primer sequences are shown in Table 1.

The endogenous control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were amplified and utilized to normalize the PD-1, CTLA-4, and LAG-3 gene mRNA levels. Sequences of GAPDH primers are also given in Table 1. The primers were synthesized by Alpha DNA Ltd (Canada) and stored lyophilized until use.

Results

Total RNA was successfully extracted from all samples. The concentration of total RNA ranged from 0.15 to 22 ng/μl with a mean ± standard deviation (SD) of 7.311 ± 0.3 ng/μl in AML patients, while the concentration of total RNA ranged from 0.5 to 40 ng/μl with a mean ± SD of 10.9 ± 0.54 ng/μl in healthy controls. Results of total RNA concentration are presented in Table 2.

There was no significant difference between the concentration of the total RNA between AML patients and healthy group, $P = 0.35$.

cDNA reverse transcription

On the 2nd day of RNA extraction, cDNA reverse transcription was performed. A common primary reaction was applied since both the study gene and the housekeeping gene needed to have cDNA.

The efficiency of the cDNA concentration was evaluated through the efficiency of qPCR carried out; later on, all measures were correlated with perfect yield representing effective reverse transcription.

Results of primer optimization of glyceraldehyde 3-phosphate dehydrogenase, cytotoxic T lymphocyte antigen-4, programmed cell death protein-1, and lymphocyte activation gene-3 genes (concentration and annealing temperature)

The optimum concentration of GAPDH forward and reverse primers was 300 and 600 nM, respectively. The

Table 1: Probes used in the study

Primer/probe	Sequence (5′→3′ direction)
PD-1	
Forward	gtgcctgtgttctctgtgga
Reverse	gagcagtgtccatcctcagg
CTLA-4	
Forward	tgtgcggcaacctacatgat
Reverse	catgagctccaccttgcaga
LAG-3	
Forward	tgatctgcccagctttccag
Reverse	actgagcctcccacatctct

GAPDH=Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene), LAG-3: Lymphocyte activation gene-3

Table 2: Concentration of total RNA

Groups	Total RNA concentration (mean±SD)	Range (ng/μl)
AML patients (*n*=50)	7.311±0.3	0.15-22
Healthy control (*n*=50)	10.9±0.54	0.5-40
t-test value	31.1	0.64 (NS)
P	0.840	0.35

AML: Acute myelogenous leukemia, SD: Standard deviation, NS: Not significant

optimum concentration of forward and reverse primers for CTLA-4, PD-1, and LAG-3 was 300 nM [Table 3].

Optimization of primer concentration is an important step in any PCR reaction. In RT-PCR, the optimum concentration is calculated according to a mesh work of serial reactions containing different dilutions of the primers making one of the primers constant in concentration in each mesh. The optimal concentration is that which gives the lowest cycle threshold (Ct) value with the highest delta Rn (dRn): The magnitude of the fluorescence signal generated during the PCR at each time point. The dRn value is determined by the following formula: (Rn+) − (Rn−) (SmartCycler II Manual, 2012). Optimal primers' annealing temperature was calculated from the melting temperature (Tm) of each primer supplied in the manufacturer's instructions according to specific equations. The equation calculates the annealing temperature, which requires a primer sequence, as it requires specific amounts of nucleotides. The equation is given below:

- Melting temperature (Tm) = (A+ T)2 + (G+ C) 4
- Annealing temperature (Ta) = Tm − (2 − 5)°C.

Using the above equations, the melting points of the forward primer and reverse primer were calculated. The lowest temperature (°C) was selected by comparing the annealing temperatures of the forward and reverse primers.[59]

Results of quantitative real-time PCR

To evaluate the RT-PCR used in the current experiment, SYBR, a fluorescent dye that recognizes any double-stranded DNA, including cDNA, was used and the amplification was recorded as the Ct value. A lower Ct value indicates higher copies of the target and vice versa. In terms of gene expression, high Ct values indicate low gene expression and low Ct value indicates a high gene expression.[60]

Real-time PCR quantification of glyceraldehyde 3-phosphate dehydrogenase expression

The range of Ct value for *GAPDH* in acute myeloid patients group was 19.85–30.08 with a mean ± SD (26.059 ± 0.713), while in apparently healthy group, it ranged from 17.12 to 21.00 with a mean ± SD (18.542 ± 0.386). A significant difference was found in between these groups regarding the mean Ct value of GAPDH ($P < 0.01$) as in [Table 4].

The underlying presumption in molecular research of the use of housekeeping genes is that their expression in the cells or tissues under investigation remains constant.[2] GAPDH is one of the housekeeping genes most frequently used in gene expression comparisons. To investigate the value of GAPDH in the human tissues as a housekeeping gene, GAPDH mRNA expression was measured in a panel of 72 different types of pathologically normal human tissue.[9,61] Studies with qRT-PCR have calculated the expression of 1718 genes using GAPDH as the reference gene in 72 forms of normal human tissues, according to the studies by Chen *et al*. and Wendelbo *et al*.[62,63] The use of GAPDH in the clinical studies of cervical human tissue human papillomavirus positive has appeared as a reliable standardization strategy in qRT-PCR using the NormFinder program (Aahus, Denmark). GAPDH was most variable and showed a tendency toward upregulation in the AML samples.[35]

Table 3: Concentration and annealing temperature of study genes

	Forward primer concentration (nM)	Reverse primer concentration (nM)	Annealing temperature (°C)
GAPDH	600	300	59
CTLA-4	300	300	60
PD-1	300	300	62
LAG-3	300	300	60

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, CTLA-4: Cytotoxic T lymphocytic antigen-4, PD-1: Programmed cell death 1, LAG-3: Lymphocyte activation gene-3

Table 4: Cycle threshold value of glyceraldehyde 3-phosphate dehydrogenase expression among patient and control groups (mean±standard deviation)

Group	*n*	Mean±SD of Ct value	Range	*t*-test value
Healthy control	50	18.542±0.386	17.12-21.00	8.96*
AML patient	50	26.059±0.713	19.85-30.08	

*$P<0.01$. Ct: Cycle threshold, SD: Standard deviation, AML: Acute myelogenous leukemia

Real-time PCR quantification of cytotoxic T lymphocyte-4 CTLA-4 expression

The $2^{-\Delta Ct}$ value for AML patients was 4.47 while for healthy control was 2.17. The computed ratio for gene fold expression was 2.059. Such minor differences in the expression of gene fold between the study groups make GAPDH gene a useful control factor [Table 5].

Real-time PCR quantification of programmed cell death protein expression

The programmed cell death protein (PD-1) expression of the acute myeloid leukemia patients was 16.929-fold higher than the healthy control and the $2^{-\Delta Ct}$ of the patients was 114.89 while the $2^{-\Delta Ct}$ of the healthy controls was 6.78 [Table 6].

Real-time PCR quantification of lymphocyte activating gene 3 expression

In the present study, the gene expression of LAG-3 was significantly different between patients and healthy control. The $2^{-\Delta Ct}$ of the patients was 27.78 while healthy was 2.02 and the fold of gene expression was 13.75 higher than the healthy control (1.00) [Table 7].

Patients' control differences in cluster differentiation markers

In 50 AML patients, multiparameter FCM (MFC) was performed on PB specimens at diagnosis to monitor changes in the expression of IC receptors. We assessed the expression of inhibitory (CD279 PD-1, CD157 CTLA-4, CD223 LAG-3) and stimulatory (CD28) coreceptor on CD3 T cell subsets.

Gating of lymphocytes was focused on flow cytometry (FCM) forward side scatter (FSC) and side scatter and expression of T lymphocyte in markers CD3. The lymphocyte popularization could either be defined as a separate population in the FSC diagram, where the lymphocytes displayed partial or full overlap with the dominant AML cell population CD3+ T cells (Figures 2a-f and Figures 3a-f).

The mean number of absolute T cells (CD3) in the AML patients was 4714 ± 1678 lymphocyte/μl and the mean number of absolute T cells inthe healthy controls was 119 ± 11 lymphocyte/μl [Table 8].

The mean percentage of lymphocyte (CD3) in the AML patients was 20.1 ± 2.3 and the mean of lymphocyte in the healthy controlx was 2.20 ± 0.23. The present study showed significantly different ($P < 0.01$) in the CD3 between AML patients and healthy controls [Table 9].

In the present study, the CD28 was in AML patients was 70.72 ± 3.12 while in the healthy volunteer was 3.720 ± 0.624. There was highly significant difference between patients and controls [Table 10].

In the present study, CTLA-4 expression on the T cell in the AML patients was 1.99 ± 1.60 and in the healthy volunteers was 0.782 ± 0.499. There was no significant difference between patient and healthy donors in the expression of CTLA-4 inhibitory molecules ($P > 0.05$) [Table 11].

In the current study, the mean LAG-3 expression on the T cells in AML patients was 1.688 ± 0.25 and in healthy volunteers was 2.594 ± 0.324. Further, there were no significant differences between patients and healthy donors in the expression of LAG-3 [Table 12].

In the current manuscript, the expression mean of PD1 on T cells was 33.34 ± 2.6 in the AML patients while in the healthy controls was 1.548 ± 0.360. The present study results show markedly increased expression of PD-1 in AML patients and highly significant than that in the healthy control ($P < 0.01$) [Table 13].

Estimate prognostic parameters of acute myeloid leukemia in different stages

In the course of current analysis, we examined periphreal

Table 5: Cycle threshold, Δ cycle threshold, and $2^{-\Delta Ct}$ cytotoxic T lymphocyte-4 expression among patient and control groups

Groups	Means Ct of *CTLA-4*	Means Ct of *GAPDH*	ΔCt (means Ct of *CTLA-4* - means Ct of *GAPDH*)	$2^{-\Delta Ct}$	Experimental group/ control group	Fold of gene expression
AML patients	25.795	24.08105	−0.26428	4.47	4.47/2.17	2.059
Healthy control	18.412	18.542	−0.13	2.17	2.17/2.17	1.00

AML: Acute myelogenous leukemia, Ct: Cycle threshold, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, CTLA-4: Cytotoxic T lymphocytic antigen-4

Table 6: Cycle threshold, Δ cycle threshold, and $2^{-\Delta Ct}$ value programmed cell death protein (antiapoptotic cell death 1) expression among patient and control groups

Groups	Means Ct of *PD-1* gene	Means Ct of GAPDH	ΔCt (means Ct of PD1 - means Ct of *GAPDH*)	$2^{-\Delta Ct}$	Experimental group/ control group	Fold of gene expression
AML patients	26.73	24.08105	0.68	114.89	114.89/6.786911	16.92915059
Healthy control	22.386	18.542	3.844	6.786911	6.786911/6.786911	1.00

Ct: Cycle threshold, AML: Acute myelogenous leukemia, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Table 7: Cycle threshold, Δ cycle threshold, and $2^{-\Delta Ct}$ (lymphocyte activation gene-3) expression among patient and control (mean±standard deviation)

Groups	Means Ct of *LAG-3*	Means Ct of *GAPDH*	ΔCt (means Ct of *LAG-3* - means Ct of *GAPDH*)	$2^{-\Delta Ct}$	Experimental group/ control group	Fold of gene expression
AML patients	26.42	24.08105	1.215	27.78916491	27.789164/2.02075	13.75186
Healthy control	21.442	18.542	2.9	2.02075	2.02075/2.02075	1.00

LAG-3: Lymphocyte activation gene-3, Ct: Cycle threshold, AML: Acute myelogenous leukemia, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Table 8: Absolute T cell (CD3) expression among patient and control in peripheral blood cell by flow cytometry

Marker	*n*	Mean±SE	Range	*P*	*t*-test value
Absolute T cell (CD3) patients' lymphocyte/mcL	50	4714±1678	48-52,800	0.009	2.74**
Absolute T cell (CD3) control lymphocyte/mcL	50	119±11	38-196		

**P<0.01 NS. NS: Nonsignificant, SE: Standard error

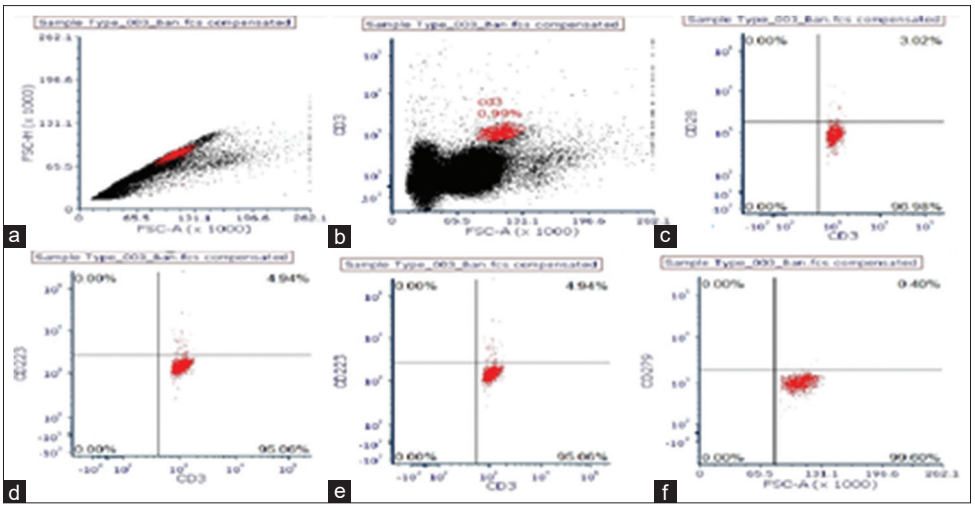

Figure 2: Flow cytometric identification of the peripheral blood healthy control and analysis of the lymphocyte population. (a and b) Independent, partly overlapping, or overlapping T lymphocyte and healthy populations. Forward side scatters and side scatters have identified the lymphocyte population of peripheral blood originating from healthy control as well as positive T lymphocyte marker CD3 staining. (c) Separating of CD28 positive from CD3 population. (d) Separation of C152 from CD3 population. (e) Separation of CD223 from CD3 population. (f) Separation of CD279 from the CD3 population

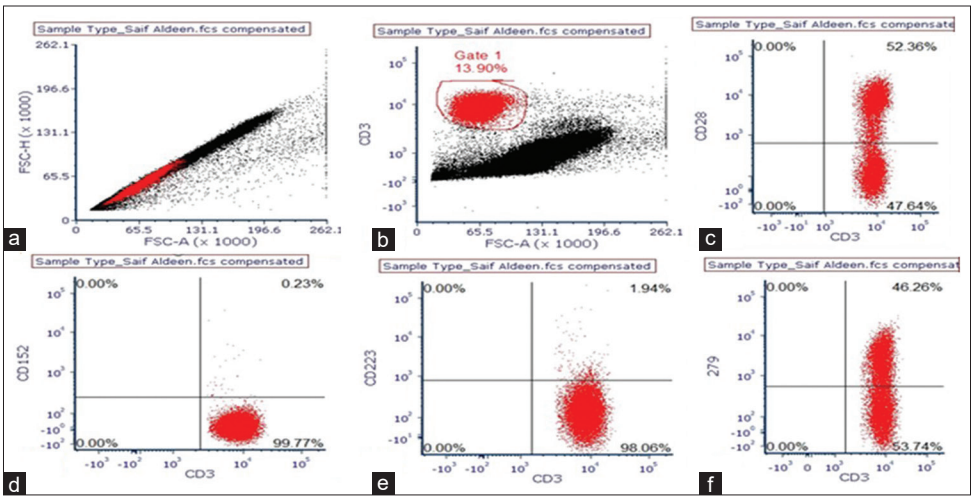

Figure 3: Flow cytometric identification of the peripheral blood acute myelogenous leukemia patients and analysis of the lymphocyte population. (a and b) Independent, partly overlapping, or overlapping T lymphocyte and acute myelogenous leukemia populations. Forward side scatters and side scatters have identified the lymphocyte population of peripheral blood originating from acute myelogenous leukemia patients as well as positive T lymphocyte marker CD3 staining. (c) Separating of CD28 positive from CD3 population. (d) Separation of C152 from CD3 population. (e) Separation of CD223 from CD3 population. (f) Separation of CD279 from the CD3 population

Table 9: CD3 (lymphocyte) expression among patient and control in peripheral blood cell by flow cytometry

Marker	*n*	Mean±SE	Range	*P*	*t*-test value
CD3 patients %	50	20.1±2.3	0.67-66.0	0.00	7.82**
CD3 control %	50	2.20±0.23	0.63-3.91		

***P*<0.01, NS. NS: Nonsignificant, SE: Standard error

Table 10: CD28 (lymphocyte) expression among patient and control in whole blood by flow cytometry

Marker	*n*	Mean±SE	Range	*P*	*t*-test value
CD28 patients	50	70.72±3.12	0.03-99.25	0.00	21.06**
CD28 control	50	3.720±0.624	0.340-11.32		

***P*<0.01, NS. NS: Nonsignificant, SE: Standard error

blood (PB) and bone marrow (BM) to measure the blast and serum samples of a total of 50 patients with newly AML patients (8%, *n* = 4), partial remission (PR) AML patients (22%, *n* = 11), AML patients in complete remission (CR, 10%, *n* = 5), refractory patients (RF, 36%, *n* = 18), and relapse patients (R, 24%, *n* = 12).

In the present manuscript, the morphological parameters (WBC and blast) and CD markers (PD-1, CTLA-4, and LAG-3 mRNA expression) were measured and estimated the level of physiological parameters (matrix metalloproteinase 2 and prostaglandin E2) in the peripheral mononuclear blood cells (PBMNCs) of a cohort of 46 treated (PR, CR, RF, and R) patients and four patients untreated yet.

The CD3 percentage expression was (20.79 ± 5.63) in relapsed patients with AML and (16.22 ± 5.12) in

refractory patients of AML, compared with newly diagnosis patients (15.14 ± 4.19) while the complete remission CR (23.92 ± 4.33) and partial remission PR (23.49 ± 4.25) were within the normal range of healthy controls (2.20 ± 0.23) [Table 14].

The expression of the CD3 in the CR and PR was higher than controls and also slightly higher than that in relapse, refractory, and newly diagnosis AML patients.

The higher percentage expression of CD28 was found in the present analysis in partial remission patients (PR) was (81.70 ± 5.03) while in relapse, refractory, newly diagnosis, and complete remission (CR) patients had the same range (62.6 ± 12.0, 68.17 ± 4.39, 66.95 ± 4.0, and 67.61 ± 7.37, respectively) compared with normal range of healthy controls (3.720 ± 0.624) [Table 14].

The CTLA 4 expression was elevated (9.33 ± 9.1) in one patient with relapse than the rest patients stages of the disease, the refractory patient was (0.4 ± 0.23) of AML, newly diagnosis patient was (0.15 ± 0.06), complete remission (CR) was (0.133 ± 0.02) and partial remission (PR) (0.755 ± 0.34) were with normal range of healthy control (0.782 ± 0.499) [Table 14].

The present study estimated that the expression of PD-1 was high in all stages of disease, i.e., relapse, refractory, CR, PR, and newly diagnosis AML patients (39.18 ± 5.4, 39.54 ± 5.6, 27.99 ± 3.9, 29.74 ± 4.86, 22.40 ± 5.8, respectively), than healthy volunteers' PD-1 expression (1.548 ± 0.360) [Table 14]. The expression percentages of the CD223 in the present data were approximately similar without any elevation in the expression in relapse (1.586 ± 073), refractory (1.43 ± 0.42), PR (2.329 ± 0.42), CR (0.83 ± 0.322), newly diagnosis (2.15 ± 1.02), and even in the healthy control (2.594 ± 0.324) [Table 14].

Table 11: CD152 (cytotoxic T lymphocyte antigen-4) expression among patient and control in hole blood by flow cytometry

CD152 (CTLA-4)	*n*	Mean±SE	Range	*P*	*t*-test value
CD152 (CTLA-4) patients	50	1.99±1.60	0.01-64.39	0.456	0.75
CD152 (CTLA-4) control	50	0.782±0.499	0.320-1.61		(NS)

P<0.05, NS. NS: Nonsignificant, SE: Standard error, CTLA-4: Cytotoxic T lymphocytic antigen-4

Table 12: CD223 (lymphocyte activation gene 3) expression among patient and control in whole blood by flow cytometry

Marker	*n*	Mean±SE	Range	*P*	*t*-test value
CD223 (LAG-3) patients	50	1.688±0.25	0.09-2.07	0.030	−2.21
CD223 (LAG-3) control	50	2.594±0.324	0.01-4.940		(NS)

P<0.05, NS. NS: Nonsignificant, SE: Standard error, LAG-3: Lymphocyte activation gene-3

Table 13: CD279 (programmed cell death protein-1 expression patient and control in whole blood by flow cytometry

Marker	*n*	Mean±SE	Range	*P*	*t*-test value
CD279 (PD1) patients	50	33.34±2.6	5.76-87.80	0.000	12.20**
CD279 (PD1) control	50	1.548±0.360	0.16-6.00		

***P*<0.01. PD1: Programmed cell death protein-1, SE: Standard error

Immunophenotyping of the T cell in the AML patients

In the current data at a cellular level, the immunophenotyping of T cells in acute myeloid leukemia by FCM analysis was $CD3^+$ $CD28^+$ $CD152^-$ $CD279^+$ $CD223^-$ in the 48 patients (96%) (relapse, refractory, PR, and also newly diagnosis), while in one (2%) relapse patient was $CD3^+$ $CD28^+$ $CD152^+$ $CD279^+$ $CD223^-$ with 95% blasts and another (2%) refractory patient was $CD3^+$ $CD28^+$ $CD152^-$ $CD279^-$ $CD223^-$ with 80% blasts.

Discussion

In this study, we found a significantly difference between AML patients and healthy controls, there was an elevated in the CTLA-4 expression higher than healthy control; this result agrees with the results by Brown *et al*. and Murata and Dalakas[59,64] also. The amount of extracted CTLA-4 and GAPDH mRNA was studied with the mentioned increase in PCR cycles.[65] The level of CTLA-4 expression was measured with RT-PCR. Relative measurements of target gene expression were calculated using the $2^{-\Delta Ct}$ method, and GAPDH was used for normalization and indicates significance in relation to control;[58] further, another study showed that CTLA-4 expression was (12.5-fold) higher than healthy control[37] also, a study in Egypt observed an increase

Table 14: Acute myelogenous leukemia patient among all parameters and stages of disease

Parameters	Control	Newly (*n*=4)	PR (*n*=11)	CR (*n*=5)	RF (*n*=18)	R (*n*=12)
CD3±SE	2.20±0.23	15.14±4.19	23.49±4.25	23.92±4.33	16.22±5.12	20.79±5.63
CD28 mean±SE	3.720±0.624	66.95±4.0	81.70±5.03	67.61±7.37	68.17±4.39	62.6±12.0
CD152±SE	0.782±0.499	0.15±0.06	0.755±0.34	0.133±0.02	0.4±0.23	9.33±9.1
CD223±SE	2.594±0.324	2.15±1.02	2.329±0.42	0.83±0.322	1.43±0.42	1.586±073
CD279±SE	1.548±0.360	22.40±5.8	29.74±4.86	27.99±3.9	39.54±5.6	39.18±5.4

SE: Standard error, PR: Partial remission, CR: Complete remission, RF: Refractory, R: Relapse

in the expression in AML patients, significantly in CTLA-4 (P = 0.005) as compared with the healthy control group.. AML patients with unfavorable prognosis also showed significant upregulation of mRNA expressions of CTLA-4 (*P* = 0.006) relative to those with favorable prognosis.[58] In previous study in Shiraz University by Ramzi *et al*.,[61] in peripheral blood mononuclear cells (PBMCs) of AML patients, they showed that the mRNA expression of CTLA-4 (5.7-fold) and CD28 (7.9-fold) increased significantly compared with healthy controls (*P* = 0.006 and 0.02, respectively).

This analysis agrees with the study by Yang *et al*.[37] Programmed death-ligand -1 (PD L1) upregulation (≥0.2-fold) was observed in 36% of samples of AML patients. In Peripheral Blood Mononuclear Cells (PBMNCs), 61 patients receiving epigenetic therapy, including 24 patients with chronic Myelodysplastic syndrome(MDS), 5 patients with chronic myeloid leukemia CML, and 32 patients with Acute myeloid leukemia (AML) and mRNA expression levels for programmed ligand -1 (PD L1), programmed ligand -2 (PD L2) were evaluated. The relative expression of mRNA was quantified using normal PBMCs (*N* = 46) as controls. Increased gene expression of CTLA-4, PD-1, PD-L2 genes in myeloid malignancies patients was also observed also another study showed the high expression of PD-1 in T cells.[31,35] Further, Schnorfeil et al. showed there were elevated in PD-1 inhibitory molecules expression (PD-1) in CD3 T cells of the AML patient in comparing with health control.[66]

The $2^{-\Delta Ct}$ of the patients was 27.78 while of healthy was 2.02 and the fold of gene expression in patients was 13.75 which is higher than that in healthy control. These results agreed with those reported by Andrews *et al*. and Radwan *et al*.[42,58] In addition, the role of these inhibitory receptors in the pathogenesis of AML is of great concern to further understand LAG-3, as coinhibitory molecules, along with other immune escape mechanisms followed by tumor cells, will diminish tumor-specific T significantly dell responses.[67] Once activating T cells, LAG-3 expression was first detected approximately 24 h after activation, peaking on the 2nd day, and then slowly declining on the 8th day. Early studies on LAG-3 showed that this expression may help differentiate Th1 cells from Th2 CD4 T cells; that is, IL-12 can effectively stimulate LAG-3 expression, and IFN-gamma blockade will reduce LAG-3 expression.[68] LAG-3 is a receptor of the IC expressed on activated/exhausted T cells. When engaged by the Class II molecules of MHC, LAG-3 regulates T cell function negatively, thus leading to tumor escape.[55] LAG-3 expression has recently been suggested as a prognostic marker in chronic lymphoid leukemia(CLL) patients, as gene expression profiling of CLL cells observed increased levels of LAG-3 expression associated with decreased treatment-free survival. A previous study showed that in secondary lymphoid tissues sample obtained from CLL patients, LAG-3 expression on CD8+ T cells has increased in comparison with peripheral blood lymphocytes (PBLs). This is consistent with the previous studies that reported increased expression of LAG-3 in CD8+ T cells infiltrating certain solid tumors as well as in a murine model of CLL;[69] further, the expression of LAG-3 was detected almost exclusively on PD-1 with CD8+ lymphocytes. Coexpression of LAG-3 with PD-1 on tumor-infiltrating lymphocytes (TILs) identifies a highly exhausted T cell population and the synergy between these inhibitory receptors in solid tumors seems to impose tumor-induced immune tolerance.[55]

PD-1 is a negative costimulatory receptor on activated T lymphocytes that counteract the activation signal provided by the TCR ligation. Similarly, PD-1 can be induced in NK cells, B cells, and monocytes. The two ligands of PD-1 are PD-L1 and PD-L2. They have different cell expression patterns. The expression of PD-L2 is mainly limited to APCs, while PD-L1 is widely expressed in the tissues and may be further stimulated by IFN-γ. Exposure to PD-L1 IFN is the main link of PD-1-mediated immune suppression. More and more evidence shows that the expression of PD-L1 on solid tumor cells can inhibit the antitumor immune response, and blocking PD-L1 can inhibit tumor growth and delay the development of mouse models. However, there is no evidence to support a functional role for this pathway in myeloid leukemia.[29] A combination of high levels of PD-1 expressed on T cell surfaces and exhaustion in lymphocyte populations when confronted by chronic viral infections such as HIV has been documented in multiple studies.[70]

Surprisingly, these results show a significant increase in total T cells in AML patients' PB compared to healthy controls, which is similar to the study by Le Dieu *et al*.,[71] while we disagreed with the study by Panoskaltsis *et al*.,[72] who revealed that the number of lymphocytes was tending to be lower than normal and T cells derived from patients with chemotherapy-induced cytopenia who have immunocompromised leukemia.[48] T cells of AML BM have huge differences in transcription profiles relative to T cells from healthy donors.[73]

The present study showed significant difference (*P* < 0.01) in the number of absolute of T cells (CD3) between AML patients and healthy controls [Table 8]. The present finding agreed with the findings by Lewis *et al*. and van Dongen *et al*.[74,75] They had reported about elevation in the expression in the CD3 lymphocyte.

The absolute lymphocyte count required for the calculation of CD3, CD4, and CD8 absolute counts was measured using the lymphocyte percentage and

the leukocyte count obtained from the full automated hematology analyzer.[76]

Further, Le Dieu *et al*., 2009[71] studied PB in newly diagnosed patients with acute myeloid leukemia (AML) to determine the effect of this disease on T cells in patients. Absolute amount of the PB T cells is increased in AML relative to healthy controls while our finding is disagreement with Williams *et al*., 2019,[27] who revealed that there was no significant difference in the calculated absolute CD3-positive cell infiltration between patients and control. The explanation of this finding return to the sample who took from BM and the present study was based on the PB.

The present study showed significant difference in the CD3 between AML patients and healthy controls. The current results agreed with the pervious studies that showed there was increase in the percentage of lymphocyte in the patients of AML compared with healthy controls.[48,62]

However, Vidriales *et al*.[77] elucidated that there were no differences between patient and control in CD3+ CD56+ for NK in BM.

A further finding revealed that T cells were both small in numbers and functional following stimulation with an anti-CD3 antibody during the periods of chemotherapy-induced leukopenia.[63]

There were highly significant differences between patient and control in the cellular expression of CD28; this result agreed with previous studies.[48,61,78,79]

T cell activation consists of two significant and necessary signals: first, antigen presentation, basically a peptide/MHC complex, via cell presenting antigen (APC) to the T cell receptor (TCR) present in the T cell. Second, the co-stimulating signal provided by APC is present by B7 molecules (B7-1 and B7-2) and their interaction with CD28 in T cells. Only when there are two signals in the reaction through the APC-T module, the positive signal and the negative signal will be activated at the same time. On the one hand, it triggers intracellular signals that lead to cytokine development, cell cycle development, and upregulation of antiapoptotic agents that cause T cell proliferation and differentiation. On the other hand, it will cause inhibitory molecules, such as CTLA-4 and other T cells such as PD1, to activate and terminate.[80,81] The pervious findings showed that more than 80% of the cells generated from the PBL samples were CD3+ CD8+, which are classified as AML-specific CTLs.[82]

In addition, Coral *et al*. demonstrated upregulating costimulatory molecules CD28 in AML patient.[83] CD28 is not expressed on the normal plasma cells PCs but is consistently and brightly expressed on malignant PCs. High proliferation activity of myeloma cells was associated with CD28 expression.[84] The explanation of this finding could be due to high expression of CD28 which compete with inhibitory molecules to interact and link tightly with their ligand on tumor surface especially with B7 receptor family. There was not a significant difference between patients and healthy donors in the expression of CTLA-4 inhibitory molecules ($P > 0.05$). The present result agreed with the result of Pistillo *et al*., 2003;[23] they observed a negative CTLA-4 surface expression profile on resting cells as the molecule did not constitutively express freshly differentiated PB, T cells, B cells, granulocytes, or CD34 stem cells.

The current results agreed with the results by Weber,[85] who showed that there was increase the expression of CTLA-4 in patient that resistance to the chemotherapy. This study revealed their was increased in expression of CTLA-4 in relapse patient compared with newly diagnosis and CR.

However, the upregulated expression (64%) of CTLA-4 on T cells was only found on one relapse case.

While the current results disagree with the results by Chen *et al*., 2020,[62] who reported that there was high expression of CTLA-4 in the CD8 compared to healthy controls, but the present result agreed with Chen *et al*.,[62] who found there was not a significant difference in the expression of CTLA-4 in CD4 T cell. These findings were tempting to speculate that CTLA-4 highly affinity to bound with CD8 than CD4 that return that CD8 were the first activated than CD4 against leukemic cells. Further, this analysis disagreed with Ramzi *et al*.,[61] who showed that there were significant differences in expression of CD8+ CD152+ in AML patients compared to healthy volunteers using RT-PCR while the current study used FCM analysis to measure the expression on the T cells.

Such a discrepancy may suggest that CTLA-4 alterations are involved in different exhausted T cell subsets in AML; however, to validate these results, further study with a larger cohort of AML samples is required.

In addition, the present results contrast strongly with the research performed by Chen *et al*.[62] The current study showed that cases of AML had the lowest expression of CTLA-4, while their analysis showed that CTLA-4 was strongly expressed in AML, taking into consideration chemotherapeutic approaches which may have a huge effect in decrease the expression on CTLA-4 in AML patients.

Through examining AML patient samples, scientists observed that 80% of AML samples checked for diagnosis constitutively expressed CTLA-4 and that CTLA-4 blockade may cause leukemic cell killing via apoptosis.[22]

In the current manuscript, the results agreed with Chen *et al*.,[62] who did not find a statistically significant difference in the numbers of LAG-3+ CD3+, CD4+, and CD8+ T cells subset. Such findings suggest that LAG-3 modifications may not be a main factor in AML immune suppression, at least not for all subsets of T cells.

The findings of FCM analyses showed no difference between the percentages of LAG-3 and PD-1 double-positive T cells of the PB of patients with breast cancer from various clinical stages or molecular subtypes ($P > 0.05$),[86] which disagree with the studies by Chen *et al*. and Dama *et al*.[32,87]

LAG-3 has been identified as an inhibitory receptor of the IC expressed on CD4+ and CD8+ T cells, Treg cells, and dendritic cells. Based on its structural similarity, LAG-3 competes for binding to MHC Class II with CD4 and suppresses T cell activity with CD4+. In addition, LAG-3 interlinks with CD3 to inhibit T cell proliferation and cytokine production.[88]

LAG-3 expression on TILs has been shown in various solid malignancies, and combined anti-LAG-3 and anti-PD-1 therapy has resulted in improved antitumor immunity in colon murine and ovarian.[89]

Most specifically, as regards the altered expression of LAG-3 and CTLA-4, LAG-3 was upregulated not only in T cells but also in leukemia cells. CLL cells have been reported to be able to produce and secrete LAG-3, which has been correlated with shorter survival free from medication. The reason for this finding may include (1) soluble LAG-3 improves leukemic cell activation and prevents leukemic cell apoptosis by interacting with MHC Class II and (2) MHC Class II+ CLL cells may impact LAG-3+ T cells and induce immune fatigue on the CLL microenvironment.[69] However, it would be interesting to analyze in the future if such an anomalous expression of LAG-3 occurs in other forms of leukemia cells.

The present analysis agreed with Wang *et al*.,[79] who used FCM analysis revealed that the frequency of levels of PD-1 on T cells in AML patients was significantly higher than those from healthy controls also the present analysis agreed with Schnorfeil *et al*., Weber, and Martins *et al*.[14,66,85]

Further, the current results were agreed with previous reports that revealed PD-1+ T cells in AML, where both CD4 and CD8 were shown to have significantly increased.[32,62,87] Also, another study that showed a significant increase of the PD1 expression in CD3 of AML patient and also increases in the CD8 while there was no significant increase in the CD4.[33,35]

The present analysis disagreed with the study by Scutti and Luiz,[2] who revealed low expression of PD-1 on non-Hodgkin's lymphoma and B cell lymphomas.

PD-L1 is one of the intrinsic receptors of immune cells responsible for controlling the T cell activation cycle. It is one of the pathways that cancer cells upregulate to avoid the development of antitumor effector T cells and enable the tumor to escape the immune system.[90]

The present observation was in line with Le Dieu *et al*.,[71] who showed a significant increase in total T cells in untreated AML patients PB, compared with healthy volunteers. Further, Maraninchi *et al*.[91] noticed a marked rise in activated T cells (represented by cells CD3 and $CD3^+$ $CD25^+$ and NK cells (represented by expression CD56). During therapy, NK cells even increased.

The CD3-positive cell subset was substantially more prevalent in BM aspirates (BMAs) from patients with AML health doners (HDs vs. new AML vs. relapsed AML: 60.3% vs. 78% vs. 81.1%);[27] the differences in the percentages return to the sample who used from BMA while the present study used PB. Patients in CR from AML have been reported to have reduced CD4+ T cells, while proliferative function remained normal.[21]

The higher percent of CD28 expression was found in patient with partial remission , while in relapse,refractory, newly diagnosis, and CR patients were almost in the same range. The main positive costimulatory receptor on T cells is CD28, and its ligands are the B7 family of molecules CD80 and CD86, which are expressed overwhelmingly on activated APCs.[92] In the previous study, patients receiving induction chemotherapy were collected and administered with hematologic nadir, 10–12 days after chemotherapy, in all cases as the source of cells for expansion of CD3/CD28 T cells.[93]

The current result was coordinated with the results of Robillard *et al*., 1998. It has been found that 41% of patients with multiple myeloma express CD28 on Bone marrow plasma cell (BMPCs), but its expression is higher in recurrent myeloma. It was found that 59% of patients with myelinated recurrence and 93% of patients with extramedullary recurrence were highly sensitive to CD28 expression.[84]

The above data provide a working model in which CD28 improves and CTLA-4 inhibits T cell responses

while both interact with the same ligands, which poses the essential problem of how T cells want to use CD28 and CTLA-4; there are three possibilities – first, ligand competition; second, CTLA-4 signaling; at last, regulatory cell cytokines hypothesis;[93] the present data showed increase in the expression of CD28 while decrease in the expression of CTLA-4 that confirmed and reflected the hypothesis of previous studies.

The expression of CTLA-4 has been similar in both untreated and chemoresistant samples. CTLA-4 transduced an apoptotic signal on involvement in chemoresistant AML cells with its recombinant ligands r-CD80 and r-CD86, inducing an average of 71% apoptotic cells at the maximum concentration, respectively. CTLA-4 was expressed similarly in patients with CR or with patients no response to therapy[22] while Liao *et al*., 2019 reported that the level of expression of CTLA-4 on effector CD4+ and CD8+ T cells in BMA samples was increased from patients who displayed no response compared to responders to the drug.[94]

The current study agreed with previos studies by Schnorfeil *et al*., Gbolahan *et al*., and Haroun *et al*.[66,95,96] in which the relapse patients have same result of PD-1, while another study could not show the expression of PD-1 on either peripheral blood or BM blasts in the children.[97]

Daver *et al*., 2016[98] had been examined the PD-1 expression of 74 AML patients (36 untreated, 38 again) and 8 safety controls using BM perfusion for a MFC showed that all subpopulations of T cells (CD4 T cells), increased regulatory CD4 T cells, and CD8 T cells significantly increased the expression of PD-1 in untreated and relapsed AML patients compared to healthy controls. In the reflex AML group, PD-1 expression was higher in CD4 T cells and CD8 T cells; in the untreated AML population, PD-1 expression is strongest in CD4 Treg cells. The expression of PD-1 on the BM cells was higher in relapsed AML relative to newly diagnosed AML or healthy donors, progressive exhaustion of T cells with more advanced AML.[80] At the time of diagnosis, the level of PD-1+ CD4+ T cells in patients was higher than in CR patients as well as PD-1+ CD8+ T cells, even though this is not significant in the latter case,[87] further, increased proportion of CD8+ PD-1 T BM cells were reported in a cohort of 22 newly diagnosed AML patients.[33] The expression percentages of the LAG-3 (CD223) in the present data were approximately similar without any elevated in expression in all stages. The current finding agreed with the one by Abdelhakim *et al*.,[99] who revealed that the percentage of LAG-3 was not change after induction of chemotherapy in the AML patients.

Similar patterns have been found for PD-1-positive/LAG-3-positive/CD8-positive T cells (HD vs. newly AML vs. recurrent AML: 2.71%, 4.69%, and 8.98%, respectively) and PD-1-positive/LAG-3-positive/CD4-positive T effector cells (HD vs. new AML vs. recurrent AML: 2.71%, 14.3%, and 13.5%).[27]

The present result revealed the dominant of PD1 receptor CD279$^+$ in (96%) of Iraqi AML patient which is responsible for resistant the chemotherapy and suggested to use PD-1 inhibitory blockade treatment with chemotherapy.

Our results at the genetic level by real-time - polymerase chain reaction (RT PCR) on the AML patients showed increased expression of PD 1 CD279 was (16.92 fold than normal) and LAG 3 CD223 expression was (13.75 fold than normal) while the expression of CTLA 4 CD152 was (2.05 fold than normal) not much elevated in our case which are similar to the result of FCM analysis. These results above confirmed that PD-1 was the main inhibitory molecule in Iraqi AML patients.

The current data agreed with the previous studies that demonstrated the dysregulation of immune systems has been well documented in AML. Immunophenotyping of T cells by FCM revealed aberrant trends of T cell activation, decreased concentration of regulating T cells, and overexpression of inhibitory immune control points such as PD-1 in up to 35% of primary AML patient samples with higher concentrations reported in patients with relapse.[100,101]

Conclusion

Most AML patients had high cellular expression of T cell CD3 and CD28. Although there was increasing gene expression of PD-1 and LAG-3 in T cell as well as cellular expression of CD279 PD-1 but not CD223 LAG-3, while gene expression of CTLA-4 had slightly increased and cellular expression of CD152 CTLA-4, but not significantly.

In the present manuscript, there was an increase of WBC and blast with progression of the disease; further, there was increased cellular expression of PD 1 CD279 in the relapse and refectory patients than the CR and PR while CD3, CD28, and CD223 LAG 3. Eventually, the immunophenotyping of 48 from 50 patients (96%) of the present study was CD3$^+$ CD28$^+$ CD152 – CD279$^+$ CD223$^-$.

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Conflicts of interest

There are no conflicts of interest.

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