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RESEARCH

# The effects of bone marrow humoral components of B-cell acute lymphoblastic leukemia patients on natural killer cell suppression

**Ilhan Tahrali[1,2] · Esra Yucel[3] · Emine Turkkan[4] · Ali Aycicek[5] · Aysegul Unuvar[6] · Suzan Cinar[1] · Gunnur Deniz[1]**

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## Abstract
B-cell acute lymphoblastic leukemia (B-ALL) is the most common form of cancer diagnosed in children. While the majority of patients survive with conventional treatment, chemotherapeutic agents have adverse effects and the potential for relapse persists even after full recovery. Given their pivotal function in anti-cancer immunity, there has been a surge in research exploring the potential of natural killer (NK) cells in immunotherapy, which has emerged as a promising avenue for treating leukemia. Nevertheless, the efficacy of NK cell immunotherapy is less pronounced than expected, which suggests the external conditions that affect NK cell functions after the administration to patients with leukemia. In this study, the effects of humoral components in the bone marrow humoral components of B-ALL patients on healthy NK cells were investigated. Healthy peripheral blood mononuclear cells were cultured with and without bone marrow-derived plasma samples of B-ALL patients. The expression of PD-1 and IL-10 were found to be increased whereas the proliferative capacities of NK cells were found to be decreased at the presence of B-ALL plasma samples. Moreover, high IL-10 versus low IL-18 levels were detected in bone marrow plasma samples of B-ALL patients. These findings indicate that humoral components in the bone marrow of B-ALL patients exert a suppressive effect on NK cell functions.

**Keywords** B-cell acute lymphoblastic leukemia · Natural killer cells · Bone marrow · PD-1 · IL-10

## Introduction

Acute lymphoblastic leukemia (ALL) is a hematological malignancy that occurs as a result of uncontrolled proliferation of lymphoid precursors in bone marrow (BM), blood, and extramedullary tissues [1]. ALL is the most common cancer in childhood [2, 3], and B-ALL accounts for the majority of whole ALL patients with a rate of more than 75% [4]. Although 5-year survival rate of pediatric cases is around 90% with appropriate treatment, relapse might occur in some patients who respond to the treatment [5, 6]. In addition, the administration of chemotherapy, with its attendant adverse effects, has a deleterious impact on the quality of life of patients. Furthermore, even children who fully recover are more susceptible to subsequent morbidity and mortality due to late adverse effects from the therapy, in comparison with healthy children [7–9]. Studies are carried out on different treatment methods for reducing the risks and side effects of chemotherapy, which include immunotherapy applications such as monoclonal antibodies, antibody–drug conjugates, and chimeric antigen receptor (CAR)-T cell application [10]. However, due to the side effects of these methods on

✉ Gunnur Deniz
gdeniz@istanbul.edu.tr

[1] Department of Immunology, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Türkiye

[2] Department of Pharmaceutical Microbiology, School of Pharmacy, Istanbul Medipol University, Istanbul, Türkiye

[3] Division of Pediatric Allergy and Immunology, Department of Pediatrics, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye

[4] Istanbul Okmeydani Health Application and Research Center, Pediatric Hematology and Oncology, University of Health Sciences, Istanbul, Türkiye

[5] Department of Pediatric Hematology and Oncology, Çam and Sakura City Hospital, Istanbul, Türkiye

[6] Division of Pediatric Hematology and Oncology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye

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normal cells, different treatment options are being investigated including natural killer (NK) cell therapy.

NK cells are large granular cells of innate immunity developing from hematopoietic progenitor cells in BM [11]. NK cells, lacking CD3 but expressing CD16 and CD56 molecules, are divided into two main subgroups $CD3^{-}CD16^{+}CD56^{dim}$ and $CD3^{-}CD16^{-}CD56^{bright}$ cells. While $CD3^{-}CD16^{+}CD56^{dim}$ NK cell subset exerts a cytolytic effect via perforin and granzyme molecules released onto target cell following activation, $CD3^{-}CD16^{-}CD56^{bright}$ NK cells are responsible for cytokine production in response to different stimuli. While activated NK cells have a lethal effect on virus-infected or malignant cells with the cytotoxic molecules in their granules, they play a role in immune regulation releasing various cytokines [12]. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α are among the most prominent cytokines produced by NK cells. IFN-γ is one of the major potent effector cytokines secreted by NK cells and plays a pivotal role in antiviral, antibacterial, and antitumor activity [13]. IFN-γ, a pro-inflammatory cytokine with multifaceted effects, interacts with macrophages, immunosuppressive myeloid cells, B lymphocytes, and stromal cells within the tumor microenvironment [14, 15], and is known to induce apoptosis of tumor cells by exerting a cytotoxic effect in conjunction with perforin and granzyme molecules [16]. IFN-γ production by NK cells is induced primarily by IL-12 produced by monocytes/macrophages as one of the initial host responses to infection [17]. IL-12, which is a potent inducer of IFN-γ production, also induces the production of TNF-α, a pro-inflammatory cytokine which first was identified as playing a role in suppressing the proliferation of tumor cells and inducing tumor regression [18]. However, there are studies demonstrating TNF-α is involved in stimulating tumor cell growth and proliferation, invasion, metastasis, and tumor angiogenesis [19]. Studies in various cancers have shown high serum TNF-α levels. It is therefore hypothesized that TNF-α may serve as a potential biomarker for cancer risk, response to treatment, and prognosis [18].

In addition to proinflammatory cytokines, NK cells also secrete a multitude of other molecules that regulate the immune system, including immune regulatory cytokines such as IL-5, IL-10, IL-13, and the growth factor GM-CSF [20]. IL-10, an anti-inflammatory cytokine, has the capability of suppressing pro-inflammatory cytokine production (especially IFN-γ) and proliferation of Th1 cells [21]. IL-10 has been demonstrated to be involved in the development of cancer and the evasion of immune responses by cancer cells. Besides solid tumors, circulating IL-10 levels were found to be higher in hematological cancers compared to healthy individuals. Given its elevated levels in a range of cancers, IL-10 has been proposed as a prognostic biomarker [22].

A substantial body of evidence suggests that IL-10, in conjunction with PD-1, exerts a suppressive influence on anti-tumoral immune responses. PD-1 is an immune-checkpoint molecule initially identified in T lymphocytes and recognized as an immune regulatory molecule responsible for limiting excessive immune responses and preventing immune-mediated damage [23]. Elevated PD-1 expression as a result of prolonged exposure of tumor-infiltrating T cells to tumor antigens and to immune suppressive microenvironment is known to cause depletion in cellular functions [24]. Subsequent to ligand binding, the proliferative and cytolytic activities of PD-1-expressing cells are inhibited. PD-1 ligands are expressed by many cancer cells and in tumor microenvironment, and PD-1 expression is increased by various inflammatory stimuli in the microenvironment [25]. Therefore, drugs which prevent the interaction between PD-1 and its ligands have been thought to be used in tumor immunotherapy [26]. It was initially postulated that the blocking of the PD-1/PD-L1 axis would only result in the unleashing of the T cell response, due to the lack of PD-1 expression on NK cells of healthy subjects. Nevertheless, it has been demonstrated that the expression of PD-1 on NK cells is markedly elevated in certain types of cancer. These studies revealed that the expression of PD-1 on NK cells, even at low levels, has a suppressive effect on the anti-tumor activity of NK cells. Furthermore, they demonstrated a correlation between higher PD-1 expression on NK cells and poorer prognostic factors in patients [27–29]. In the light of these findings regarding the presence of PD-1-expressing NK cells in cancer patients, it is postulated that anti-PD-1/PD-1L therapy may also enhance the anti-cancer responses of NK cells in a manner analogous to that observed with T cells [25].

NK cell functions are known to be impaired in different leukemia types, depending on the disease severity [30, 31]. It has been demonstrated that the presence of NK cells in the BM of ALL patients is associated with improved survival outcomes, and an increase in BM-NK cells in pediatric ALL patients is linked to a more favorable prognosis [32]. In addition to its anti-leukemia function, studies are carried out to recover NK cell deficiency in leukemia patients via NK cell immunotherapy due to its low cytotoxic effect on healthy cells. A number of studies have demonstrated a correlation between the reactivity of donor NK cells against leukemia cells and long-term disease-free survival. Furthermore, adoptive NK cell therapy has been shown to be a safe and effective treatment for patients with active acute myeloid leukemia (AML), resulting in short remissions [22, 33]. Donor NK cells were demonstrated to be expanded and persisted with anti-leukemic activity in the majority of patients with relapsed AML following hematopoietic cell transplantation [34].

Nevertheless, some studies have postulated that NK cell therapy may have limited efficacy in patients with AML, although it does not elicit any adverse effects [35, 36]. It

is established that tumor stromal cells can influence the secretion of effector cytokines by NK cells [37]. The lack of complete success in healthy NK cell functions following administration to leukemia patients suggests the existence of a suppressive effect of the leukemia microenvironment on healthy NK cells.

Given the high prevalence of B-ALL in children, it is crucial to implement novel treatment approaches that are both highly efficacious and devoid of life-threatening adverse effects. Among the available treatment options for B-ALL, CAR-NK cell therapy seems to be a promising avenue that merits further investigation [21]. Nevertheless, several challenges remain to be addressed to enhance the efficacy of CAR-NK cell therapy. For instance, the identification of suitable NK cell subsets is essential for the targeted expansion of CAR-NK cells. The optimal configuration of CARs to stimulate the activation, proliferation, and functionality of NK cells remains to be elucidated. In addition, NK cells are unable to survive for an extended period within the body following their administration [22]. Therefore, it is therefore crucial to ascertain the factors that contribute to the efficacy of NK cell immunotherapy in B-ALL.

The objective of this study is to elucidate the impact of the BM microenvironment on NK cell phenotype and functions in the context of humoral components in pediatric B-ALL patients. To this end, the expression of the PD-1 molecule, the capacity for intracellular cytokine production, and the proliferative potential of healthy peripheral $CD16^{+}CD56^{dim}$ and $CD16^{-}CD56^{bright}$ NK cell subgroups were assessed in the presence or absence of BM-plasma of patients with B-ALL. The findings of this study are intended to inform the optimization of NK cell-based immunotherapy for leukemia.

## Materials and methods

### Study groups

Newly diagnosed and untreated pediatric common B-ALL patients ($n=22$) with $CD10^{+}CD19^{+}$ blast phenotype according to the AIEOP-BFM subclassification [33] as well as pediatric patients evaluated with the suspicion of acute leukemia but had no pathology in terms of ALL and AML ($n=18$) were included in this study. BM samples from both patient groups were provided by the Division of Pediatric Hematology and Oncology, Istanbul University Istanbul Faculty of Medicine, Istanbul Prof. Dr. Cemil Taşçıoğlu City Hospital and Başakşehir Çam & Sakura City Hospital. Demographical data of patient groups are shown in Table 1.

For cell culture studies, pre-pubertal children ($n=10$) who did not have any health issues and did not use medication were also included in the study. Peripheral blood samples of healthy children were provided by the Department of Pediatrics of Istanbul Faculty of Medicine. An approval of Istanbul Faculty of Medicine—Clinical Research Ethics Committee was obtained in compliance with the Helsinki Declaration.

### Immunophenotyping of BM samples

To identify B-ALL patients and patient control groups, BM samples aspirated from patients into the EDTA tubes were processed for diagnostic immunophenotyping via flow cytometry. Prior to staining, cell count was conducted on bone marrow samples using an auto-hematology analyzer (Rayto, RT-7600, China). BM samples were stained with monoclonal antibodies (mAbs): anti-CD7 PE, -CD10 PE/Cy7, -CD19 APC, -CD34 FITC, -CD33 BV421 and -CD45 BV510 (all from BD Biosciences, San Jose, USA). Following the lysis of erythrocytes with FACS Lysing solution (BD Biosciences, San Jose, USA), samples were washed with PBS and measured on a NovoCyte (ACEA Biosciences, USA) flow cytometer running NovoExpress software.

### Separation of BM-plasma samples

In order to separate the plasma, BM specimens of patient groups were centrifuged at 3000 rpm for 5 min and cells were precipitated. Plasma samples collected and centrifuged twice for 10 min were aliquoted and stored at −20 °C

**Table 1** Demographical data of patient groups

| | B-ALL ($n=22$) | Patient control group ($n=18$) |
|---|---|---|
| Gender | 13 male–9 female | 11 male–7 female |
| Mean age (min–max) | 4.3 (1–13) | 7.3 (1–17) |
| Diagnosis | B-ALL ($CD10^{+}CD19^{+}$ blast phenotype) | Viral infection<br>Autoimmune hemolytic anemia<br>Immune thrombocytopenic purpura<br>Thrombocytopenia<br>Bicytopenia |
| CNS involvement | No | - |

until used. All procedures were carried out under sterile conditions.

## Immunophenotyping of peripheral blood samples of healthy children

For cell culture assays, mononuclear cells were isolated from heparinized peripheral blood samples of healthy children. Before the isolation, the percentages of T, B, NK, and NKT cells were determined by flow cytometry. Blood samples were stained with BD Multitest 4-color mAb cocktails: anti-CD3 FITC / CD4 APC / CD8 PE / CD45 PerCP and anti-CD3 FITC / CD16/CD56 PE / CD19 APC / CD45 PerCP (all from BD Biosciences, San Jose, USA). After the incubation, erythrocytes were lysed with FACS Lysing solution (BD Biosciences, San Jose, USA). Samples washed with PBS were measured by a FACSCalibur (BD, San Jose, USA) flow cytometer and analyzed with CellQuest software.

## Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples of healthy children by gradient centrifugation method. The blood samples were diluted with PBS and spread onto Lymphocyte Separation Medium (density: 1.077 g/ml) (Capricorn Scientific, Ebsdorfergrund, Germany). Following the centrifugation at 2000 rpm for 20 min, the interphase was collected and washed twice with PBS. Supernatant was removed and cells were resuspended in 1 ml complete RPMI-1640 medium (Gibco, Paisley, UK) which lacked fetal bovine serum (FBS). PBMCs were freshly used for culture assays.

## Culture of PBMCs with BM-plasma samples

In order to determine the effects of BM-plasma content of B-ALL patients on healthy NK cells, PBMCs were cultured with plasma samples obtained from BM specimens of B-ALL patients as well as patient control group. Frozen plasma samples were thawed and filtered through a 0.22-μm sterile syringe filter to remove cellular debris. A total of $1 \times 10^6$ cells/ml of peripheral blood mononuclear cells (PBMCs) were seeded into three separate wells of culture plates in FBS-free RPMI-1640 medium. B-ALL and control patient plasma samples, and FBS alone (as the control condition), were added to PBMCs at 10% of total volume. Two distinct culture assays were constructed to examine the alterations in the frequencies, immunophenotypes, and functions of NK cell subsets, as mentioned below.

## Determining the intracellular cytokines of NK cell subsets

PBMCs ($1 \times 10^6$ cells) together with BM-plasma of patient groups as well as FBS were stimulated with hrIL-12(p70) (15 ng/ml) (Biolegend, San Diego, USA) and cultured at 37 °C with 5% $CO_2$ incubator for 24 h to measure the intracellular cytokine content of NK cell subsets. Brefeldin A (2.5 mg/ml) (Biolegend, San Diego, USA) was added at the last hours. After the culture, PBMCs harvested and washed with PBS were stained and incubated with anti-CD3 BV785, -CD16 PE, -PD-1 Alexa Fluor 700 (all from Biolegend, San Diego, USA) as well as -CD56 PE/CF594 (BD Biosciences, San Jose, USA) mAbs for labeling NK cell subsets prior to the intracellular staining. After washing stained cells with PBS, Fixation/Permeabilization kit (Abcam, Cambridge, UK) was used in accordance with the manufacturer's protocol to detect the intracellular cytokines. Simply, cells were fixed and washed with PBS and then permeabilized together with the addition of anti-IFN-γ PE/Cy7, -IL-10 BV421, and -TNF-α APC/Cy7 mAbs (all from Biolegend, San Diego, USA). After centrifugation with PBS, samples were evaluated by a Cytek Aurora (Cytek Biosciences, California, USA) spectral flow cytometer running SpectroFlo software.

## Measuring the proliferation of NK cell subsets

To determine the effects of BM-plasma content on NK cell proliferation, a cellular method was performed based on staining cells with Carboxyfluorescein Succinimidyl Ester (CFSE), a fluorescent dye staining the cytoplasm, and evaluating the fluorescence of divided cells. To this end, 1 μl of CFSE (5 mM) prepared with the CellTrace™ CFSE Proliferation Kit (ThermoFisher Scientific, USA), was added to PBMCs suspended in FBS-free RPMI-1640 and incubated at +4 °C for 6 min. After washing with PBS, supernatants were removed and cells were resuspended with FBS-free RPMI-1640 medium.

PBMCs ($5 \times 10^5$) labeled with CFSE were cultured with plasma samples from B-ALL and control groups, as well as FBS alone, at a ratio of 10% of the total volume. They were then completed with FBS-free RPMI-1640 to a final volume of one ml. CFSE-stained cells were stimulated with Phytohemagglutinin (PHA) (5 μl/ml) (ThermoFisher Scientific, USA) and cultured at 37 °C with 5% $CO_2$ incubator for 72 h. After culturing, the cells were harvested and stained with anti-CD3 PE/Cy5 (BD Biosciences, San Jose, USA), -CD16 PE/Cy7 (Biolegend, San Diego, USA), and -CD56 PE/CF594 (BD Biosciences, San Jose, USA) mAbs and incubated. Samples were evaluated with Cytek Aurora (Cytek Biosciences, California, USA) spectral flow cytometer running SpectroFlo software.

### Determination of cytokine levels in BM-plasma samples

The cytokine levels in BM-plasma samples were investigated by multiplex assay (LEGENDplex kit, Biolegend, USA), a bead-based flow cytometric method quantifies multiple soluble molecules simultaneously. IL-1β, IL-6, IL-10, IL-18, IFN-α2, IFN-β, IFN-γ, GM-CSF, and TNF-α levels were detected in BM-plasma samples of B-ALL and control group. The staining steps of the samples were carried out in accordance with the manufacturers' protocol. Stained samples were measured on NovoCyte (ACEA Biosciences, USA) flow cytometry, and cytokine levels were detected with the LEGENDplex Data Analysis Software.

### Statistical analysis

The data were analyzed statistically with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Normality patterns of subject groups were determined with the Kolmogorov–Smirnov test. One-way ANOVA test was used for the comparison between multiple groups prior to the post hoc evaluations which were performed by Tukey's test for parametric values and the Dunnett test for non-parametric values. The comparison of binary groups with parametric distribution was made using the Independent Sample *T* test, while with non-parametric distribution was performed using the Mann–Whitney *U* test. The statistical significance limit was accepted as ($p < 0.05$). Graphs were created by GraphPad 7.0 software and statistical data were presented as mean ± standard error mean (SEM) on graphs.

## Results

### Immunophenotypes of BM specimens

BM samples of pediatric patients with suspected of acute leukemia were evaluated for definitive diagnosis by immunophenotyping with flow cytometry. The control group consisted of patients with no pathology in terms of ALL or AML. The values obtained from cell counting (Suppl. Table S1) in BM samples of B-ALL patients were statistically compared with those of the control group.

The lymphocyte and monocyte counts of B-ALL patients were found to be significantly higher ($p \leq 0.0001$, $p \leq 0.05$, respectively) than those of the control group, while granulocyte counts were observed to be lower ($p \leq 0.01$) (Fig. 1a). Similarly, the percentages of lymphocytes and monocytes in B-ALL patients were significantly increased ($p \leq 0.0001$, $p \leq 0.01$, respectively) compared to the control group, while the granulocyte frequencies were decreased ($p \leq 0.0001$) (Fig. 1b).

For BM-immunophenotyping, at least $2 \times 10^5$ cells were acquired in P1 gate excluding debris in FSC/SSC graph and $CD45^{dim/-}$ cells were gated as blast cells. The expression of CD7, CD10, CD19, CD33, and CD34 in the blast population were examined and evaluated for B-ALL diagnosis (Suppl. Fig. S2). Patients diagnosed as common B-ALL with $CD10^+CD19^+$ blast phenotype constituted the main patient group of the study, while patients with no pathology in terms of ALL and AML were considered as the patient control group.

The gating strategy of blast cells in BM samples of both patient groups is demonstrated in Fig. 1d. In addition to the blast cells, the percentages of lymphocyte, monocyte, and granulocyte populations were also determined on the FSC/CD45 graph (Suppl. Table S1). Unlike the cell count, the percentages of lymphocytes were similar between B-ALL patients and the control group, whereas monocytes exhibited a notable decline in the former ($p < 0.0001$) (Fig. 1c). Consistent with the cell count, a significant reduction in granulocyte frequencies was detected by flow cytometry in patients with B-ALL compared to control group ($p < 0.0001$).

### Immunophenotypes of healthy peripheral blood samples

Immunophenotypes of healthy peripheral blood samples were determined prior to PBMC isolation. The frequencies of $CD3^+$, $CD4^+$ and $CD8^+$ T, $CD19^+$ B, $CD3^-CD16^+CD56^+$ NK and $CD3^+CD16^+CD56^+$ NKT cells were detected by flow cytometry. The donors with no deficiency in immune cell ratios were included in the study. T, B, NK, and NKT cell percentages of healthy children were shown in Suppl. Table S3.

### PD-1 expression of NK cells culturing with B-ALL plasma samples

NK cell subsets and their PD-1 expression were determined after culturing PBMCs with BM-plasma samples of both patient groups or with FBS alone for 24 h. The gating strategy of NK cell subsets is demonstrated on a control sample cultured with FBS (Fig. 2a).

Although $CD3^-CD16^-CD56^{bright}$ NK cells tended to decrease with the addition of B-ALL plasma, no statistically significant alteration was observed in the frequencies of the two NK cell subsets across the diverse experimental conditions. (Fig. 2b). Nevertheless, the expression of PD-1 on $CD3^-CD16^+CD56^{dim}$ NK cells was markedly elevated in the presence of B-ALL plasma samples, as compared to those from the control group ($p \leq 0.01$) (Fig. 2c).

Similarly, $PD\text{-}1^+CD3^-CD16^-CD56^{bright}$ NK cells were shown to be increased in the presence of B-ALL plasma compared to the conditions involving FBS and the control

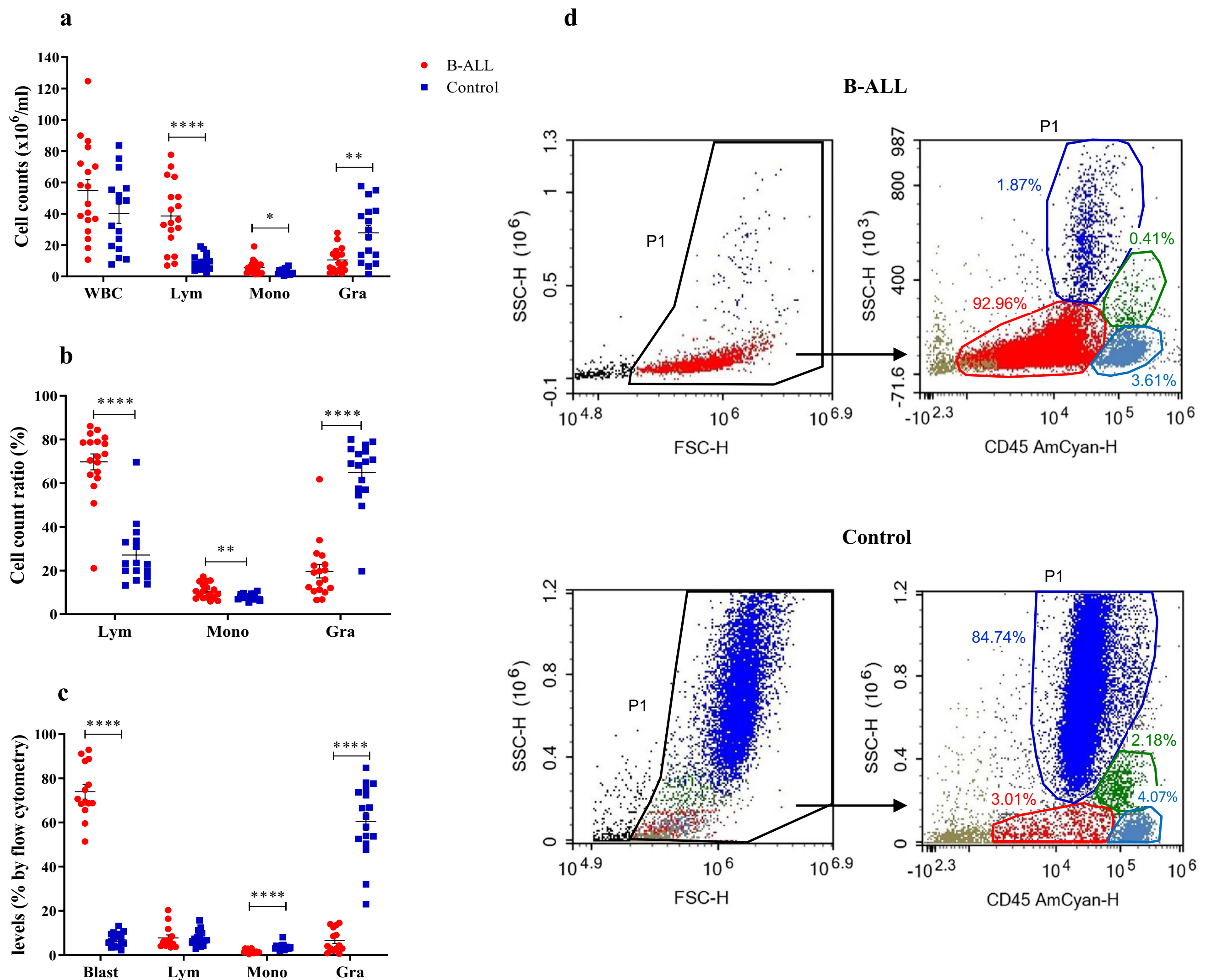


**Fig. 1** The statistical comparison of cell counts and flow cytometric representation of immune cell populations in bone marrow samples of B-ALL and control patient groups on day 0. **a** The numbers and **b** percentages of immune cells in bone marrow samples are shown. **c** The percentages of immune cell populations detected by flow cytometry. **d** The gating of blast cells as well as lymphocytes, monocytes and granulocytes of B-ALL (above) and control (below) patients are shown (*$p<0.05$; **$p\leq0.01$; ****$p\leq0.0001$) (Lym, lymphocyte; Mono, monocyte; Gra, granulocyte)

group plasma ($p\leq0.0001$, $p\leq0.0001$, respectively). On the other hand, the percentages of both NK cell subsets and their PD-1 expressions were similar between the conditions with FBS and plasma samples of control patients.

## The effects of B-ALL plasma samples on cytokine production of NK cells

In the conditions in which B-ALL plasma samples were added, there was a tendency for a decrease in IFN-γ-producing $CD3^-CD16^+CD56^{dim}$ NK cells in comparison with FBS-including conditions (Fig. 3a). The proportion of IFN-$\gamma^+CD3^-CD16^+CD56^{dim}$ NK cells in the presence of control group plasma was significantly lower than in the conditions containing FBS ($p\leq0.001$). IFN-γ production of $CD3^-CD16^-CD56^{bright}$ NK cells was found to be diminished by the addition of both B-ALL and control group plasma samples compared to FBS-containing conditions ($p\leq0.01$, $p\leq0.01$, respectively) (Fig. 3b).

In contrast to the suppressive effect on IFN-γ production observed in NK cells, the presence of B-ALL plasma resulted in a remarkable increase in IL-10-producing NK

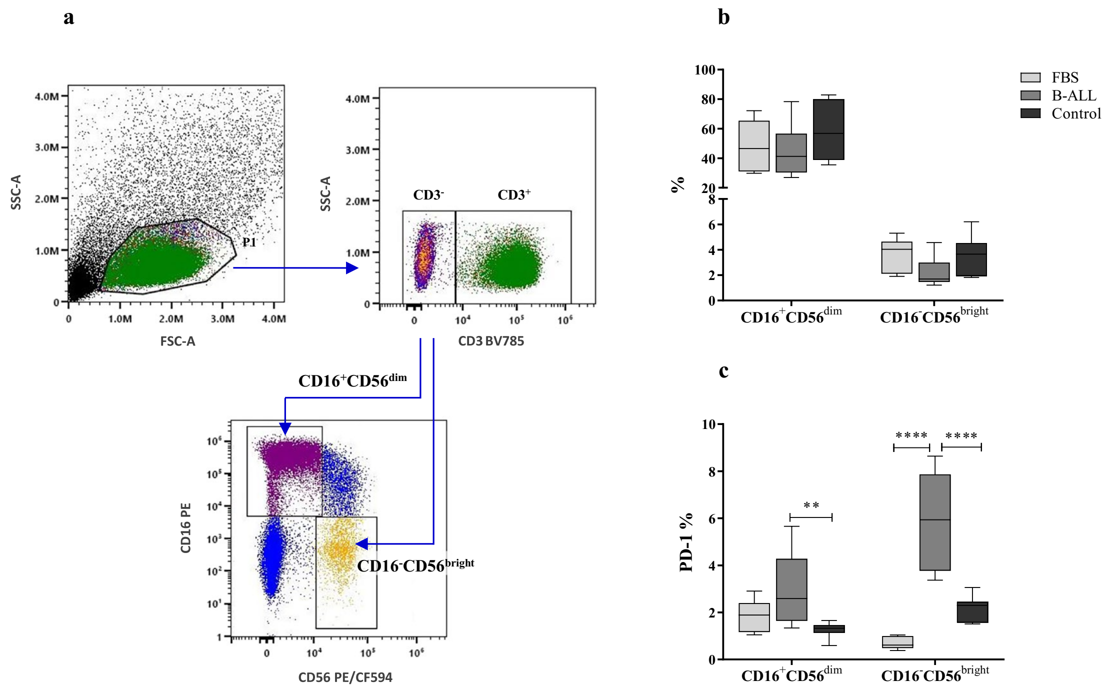


**Fig. 2** Determining NK cell subsets and their PD-1 expression by flow cytometry after 24-h cell culture at different conditions. **a** The gating strategy of NK cell subsets in peripheral blood samples of healthy donors. $CD3^-$ cells were gated in total lymphocyte gate (P1) on the FSC/SSC plot and a CD16/CD56 plot was generated within $CD3^-$ cell population to detect NK cells. Depending on CD16 and CD56 expression, NK cells were subdivided as $CD3^-CD16^+CD56^{dim}$ and $CD3^-CD16^-CD56^{bright}$ cells. **b** The frequencies of $CD3^-CD16^+CD56^{dim}$ and $CD3^-CD16^-CD56^{bright}$ NK cells, and **c** their PD-1 expression at the presence of bone marrow-plasma samples of B-ALL and control patients or FBS alone ($^{**}p \leq 0.01$; $^{****}p \leq 0.0001$)

cells. $IL\text{-}10^+CD3^-CD16^+CD56^{dim}$ NK cells were demonstrated to be significantly elevated by B-ALL plasma addition compared to the conditions with FBS and the control group's plasma ($p \leq 0.01$, $p \leq 0.01$, respectively) (Fig. 3a). Similarly, $IL\text{-}10^+CD3^-CD16^-CD56^{bright}$ NK cells were significantly increased with B-ALL plasma addition in comparison with the conditions containing FBS or control group plasma ($p \leq 0.0001$, $p \leq 0.0001$, respectively) (Fig. 3b).

The presence of B-ALL plasma samples revealed variable effects on TNF-α production of NK cells. $TNF\text{-}\alpha^+CD3^-CD16^+CD56^{dim}$ NK cells were observed to tend to decrease with B-ALL plasma addition, without a statistically significance (Fig. 3a). Conversely, B-ALL plasma supplement led to a notable elevation in the percentages of $TNF\text{-}\alpha^+CD3^-CD16^-CD56^{bright}$ NK cells compared to the conditions in which FBS, or control group plasma samples were added ($p \leq 0.05$, $p \leq 0.01$, respectively) (Fig. 3b).

## The effects of B-ALL plasma on NK cell proliferation

The proliferative capacities of NK cells were quantified in the presence of BM-plasma samples or FBS alone. The frequencies of both NK cell subsets were observed to be diminished upon B-ALL plasma addition in comparison with control groups (Fig. 4a). $CD3^-CD16^+CD56^{dim}$ NK cell frequencies were significantly lower in the presence of B-ALL plasma than those of control group's plasma samples and FBS ($p \leq 0.05$, $p \leq 0.05$, respectively). Similarly, the percentages of $CD3^-CD16^-CD56^{bright}$ NK cells were significantly decreased with B-ALL plasma addition compared to the conditions with FBS and control group plasma ($p \leq 0.0001$, $p \leq 0.001$, respectively).

Consistent with NK cell frequencies, the proliferative capacities of both NK cell subsets were reduced by B-ALL plasma addition. Control group plasma led to decrease NK cell proliferation (Fig. 4b). The proliferation of $CD3^-CD16^+CD56^{dim}$ NK cell subgroup was significantly decreased in the presence of B-ALL and control group plasma samples compared to those cultured with FBS alone ($p \leq 0.0001$, $p \leq 0.05$, respectively). A similar result was observed in the proliferation of $CD3^-CD16^-CD56^{bright}$ NK cells, which exhibited a notable decline following the addition of B-ALL and control group plasma samples, in comparison to the FBS-containing condition ($p \leq 0.0001$, $p \leq 0.05$, respectively).

## Cytokine levels in BM-plasma of patient groups

In addition to the intracellular cytokine detection, the levels of IL-1β, IL-6, IL-10, IL-18, IFN-α2, IFN-β, IFN-γ,

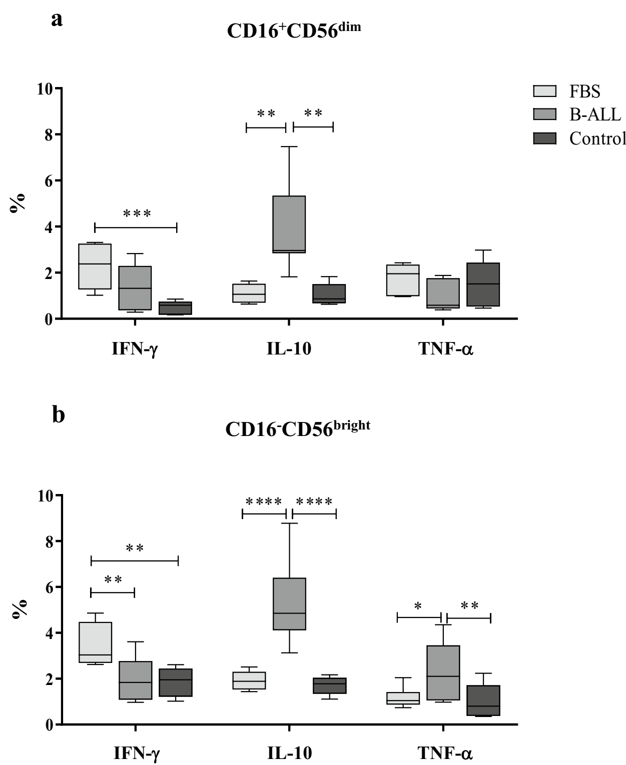


**Fig. 3** Intracellular cytokines in NK cell subsets after 24-h cell culture at different conditions. The frequencies of IFN-γ, IL-10, and TNF-α-producing NK cells were measured in response to IL-12 stimulation in **a** $CD3^{-}CD16^{+}CD56^{dim}$ and **b** $CD3^{-}CD16^{-}CD56^{bright}$ NK cells of healthy donors at the presence of bone marrow-plasma of B-ALL and control patients or FBS alone (*$p<0.05$; **$p\leq0.01$; ***$p\leq0.001$; ****$p\leq0.0001$)

GM-CSF, and TNF-α were measured in plasma samples of both patient groups by multiplex method. Similar to the intracellular IL-10 production of NK cells, IL-10 levels in BM-plasma of B-ALL patients were notably higher than the control group ($p\leq0.01$). In contrast, IL-18 levels were found to be lower in comparison to the control group ($p\leq0.05$) (Fig. 5). IFN-β levels were found to be undetectable in 50% (11/22) of B-ALL patients and 72% (13/18) of control group patients. TNF-α levels were below the detectable level in a single case of B-ALL patients (1/22), but in 28% (5/18) of the control group. GM-CSF levels were undetectable in 36% of B-ALL patients (8/22) and 50% of the control group (9/18). No significant difference was found in the measurable levels of IFN-β, TNF-α, and GM-CSF between the two patient cohorts.

## Discussion

The objective of our study was to examine the potential factors that may influence the efficacy of NK cell therapy in B-ALL. To this end, the impact of humoral components within the BM microenvironment of B-ALL patients on NK cell functions was investigated. Following 24 h of culture, the distribution of NK cell subsets remained unaltered under the various culture conditions. While the presence of BM-plasma of B-ALL patients caused a slight decrease in the frequencies of both NK cell subgroups, the main significant decrease was observed after 72 h of culture. However, the observation that the proliferative responses of NK cells were suppressed not only by B-ALL-plasma but also by plasma of control patients suggests that BM-plasma with B-ALL pathology might have a detrimental effect on NK cell survival, in addition to suppressing proliferation, which could explain the low NK cell percentages observed at 72 h. Regardless of the underlying mechanism, it is clear that the presence of B-ALL plasma exerts a suppressive effect on the expansion of the NK cell population. In this context, an investigation into the impact of BM-humoral components of B-ALL patients on NK cell apoptosis would be informative.

Similar to that of T lymphocytes, there is a recognized correlation between NK cell apoptosis and PD-1 expression.

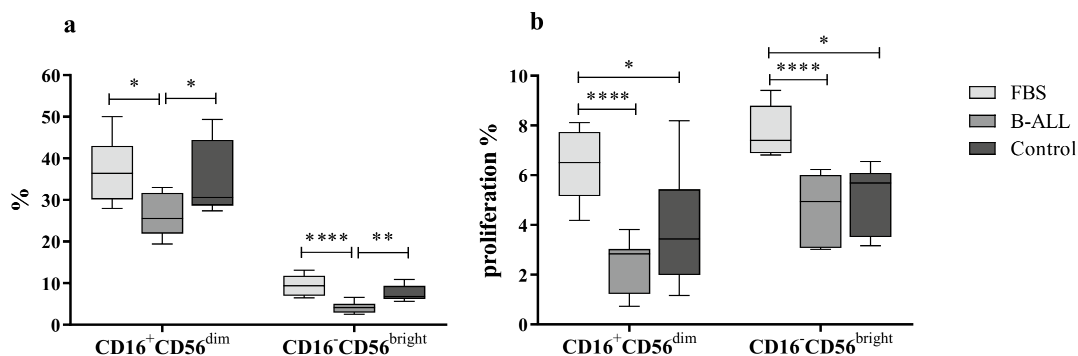


**Fig. 4** The proliferative responses of NK cell subsets after 72-h cell culture at different conditions. **a** The frequencies, and **b** proliferative responses of $CD3^{-}CD16^{+}CD56^{dim}$ and $CD3^{-}CD16^{-}CD56^{bright}$ NK cells of healthy donors in response to PHA stimulation at the presence of bone marrow-plasma of B-ALL and control patients or FBS alone (*$p<0.05$; **$p\leq0.01$; ****$p\leq0.0001$)

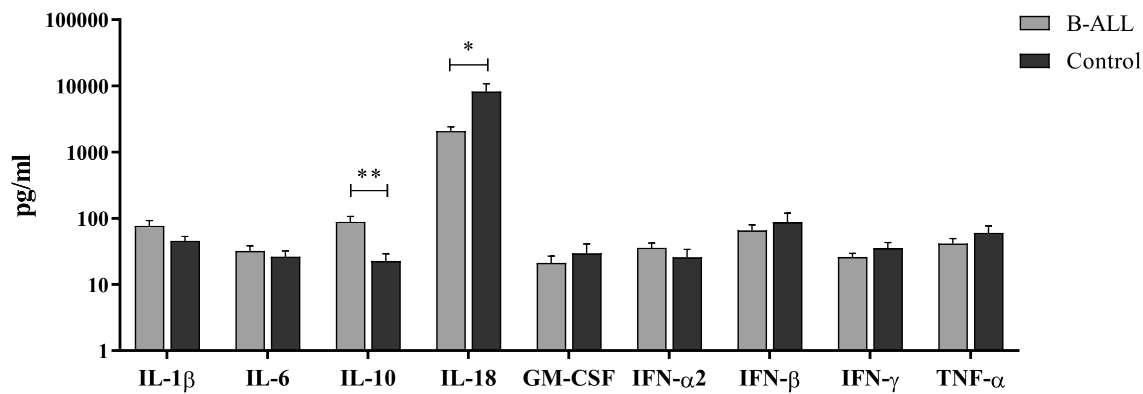


**Fig. 5** Comparison of cytokine levels in bone marrow-plasma samples of B-ALL and control patient groups (*$p<0.05$; **$p\leq0.01$)

It has been demonstrated that PD-1/PD-1L ligation promotes NK cell apoptosis, while PD-1 blockade increases NK cell survival and activity [34]. A finding from our study that is reminiscent of the link between NK cell survival and PD-1 expression was the detected elevation in PD-1 expression of NK cells, particularly in $CD3^{-}CD16^{-}CD56^{bright}$ cells, in the presence of BM-plasma with B-ALL pathology. Additionally, because of the fact that PD-1 activation results in IL-10 release [38], it is reasonable to hypothesize that increased PD-1 expression is associated with markedly elevated IL-10 production by NK cells in the presence of B-ALL plasma, as well as diminished NK cell expansion. It can be postulated that elevated IL-10 production in conjunction with increased PD-1 expression may exert an inhibitory effect on NK cell activity.

While the percentages of $IFN\text{-}\gamma^{+}CD3^{-}CD16^{-}CD56^{bright}$ NK cells were diminished upon the addition of B-ALL plasma in comparison to the FBS control group, a significant reduction in IFN-γ production was observed in both NK cell subsets with the presence of patient control plasma. This finding indicates the suppressive effect of BM-plasma content on IFN- γ production is not unique to B-ALL pathology. However, the decreased IFN- γ along with elevated IL-10 production by NK cells indicates that there are soluble components in the BM of B-ALL patients that support the production of anti-inflammatory cytokines. Another finding that lends support to this hypothesis is that plasma levels of IL-10 were higher than in the patient control group.

Furthermore, the concentration of IL-18 in B-ALL BM-plasma was markedly diminished in comparison to the control group. IL-18 is a pro-inflammatory cytokine playing role in differentiation and activation of T and NK cells. Together with IL-12, IL-18 has the ability to stimulate IFN-γ production and cytotoxic functions of $CD8^{+}$ T and NK cells [39]. The observed reduction in IFN-γ production by $CD3^{-}CD16^{-}CD56^{bright}$ NK cells in B-ALL patients may be attributed to the low IL-18 levels detected in BM-plasma samples. These findings suggest a potential transition from an inflammatory to an anti-inflammatory microenvironment, which may exert inhibitory effects on NK cells in the BM of patients with B-ALL.

TNF-α is a pleiotropic cytokine that facilitates the survival of malignant cells, evasion of immune responses, and treatment resistance. It was demonstrated that high TNF-α levels are associated with poor clinical outcomes in acute leukemia [40]. The results of our study indicate that the production of TNF-α by $CD3^{-}CD16^{-}CD56^{bright}$ NK cells is enhanced by the addition of B-ALL plasma. This finding suggests that humoral components within the BM of B-ALL patients may play a role in the growth and proliferation of blasts also by stimulating TNF-α production of NK cells.

In conclusion, this study demonstrates that the BM microenvironment of B-ALL patients exerts a suppressive effect on NK cells, with humoral factors, including cytokines, which are involved in this suppression. These findings are crucial for elucidating the factors that reduce the efficacy of NK cell-based immunotherapies in B-ALL treatment. By addressing these factors, it may be possible to potentially increase the success of these therapies, offering a more effective treatment option for patients. Such advancements could not only improve the quality of life for those adversely affected by conventional treatments but also increase survival rates in patients who are resistant to current therapies.

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**Author contribution** I.T. performed experimental assays, flow cytometric and statistical analyses, and wrote the manuscript text. E.Y. identified the healthy children who participated in the study, and provided peripheral blood samples. E.T., A.A., A.U. and S.C. diagnosed patients

with suspected ALL, performed bone marrow aspiration, and provided clinical data of the patients. G.D. ensured the collaboration of the study group and designed the study. All authors reviewed the manuscript.

**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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