Gamma-delta T cells in childhood acute lymphoblastic leukemia act as an early marker of favorable prognosis and correlate with serum HSP90.

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Summary

Acute lymphoblastic leukemia (ALL) is the leading cause of cancer related death in children despite recent advances showing improved responses to chemotherapy treatment. Gamma-delta (γδ) T cells are a recent topic of growing interest in the field of adoptive immunotherapy. These cells have also been proven to be an optimal predictor of a favorable outcome in numerous malignancies.

We evaluated subgroups of γδ T cells in the peripheral blood of 19 children with newly diagnosed ALL at the time of diagnosis and following chemotherapy induction. Due to the fact that serum HSP90 serves as a ligand for γδ T cells, we also checked the correlation between HSP90 and γδ T cells.

As a result, we found, that the number of CD8+ γδ T cells in peripheral blood at disease presentation was almost three times higher in the intermediate risk group compared to patients in high risk group. Furthermore, we observed higher percentages of the subset in younger patients at diagnosis and after induction, but not in healthy controls. We also noticed negative correlations between CD8+ γδ T cells and minimal residual disease (MRD) before chemotherapy and after induction. We showed a positive association between activated CD3+ γδ T cell and serum HSP90 at presentation.

In conclusion, our data suggests that CD8+ γδ T cells may be an early marker of favorable prognosis in childhood ALL while serum HSP90 may act as an agent activating CD3+ γδ T effector cells.

1. Introduction

Acute lymphoblastic leukemia (ALL), is the most common cancer in children, adolescents, and young adults. In the past, the prognosis was distressingly poor with only a 31% chance of 5-year survival. New diagnostic and treatment modalities have contributed to drastic improvement of the patient outcomes. Today, the 5-year survival rate of patients diagnosed with ALL is more than 80%. However, despite the somewhat promising results of conventional chemotherapy, leukemia remains the leading cause of cancer related death among children. [1] The most promising novel therapies dedicated to improving treatment results for chemo-resistant and recurrent leukemic patients are based on modulation of immune system. [2] Human gamma-delta (γδ) T cells represent a small subset of $CD3^+$ T lymphocytes (1–10%), however, these cells have been gaining interest due to their unique, antitumor properties. [3] These γδ T cells demonstrate both innate and adaptive immune properties through their primary functions which include phagocytosis and presentation of soluble antigens to alpha-beta (αβ) T cells, induction of dendritic cell (DC) maturation, and destruction of cancer-associated cells. [4] However, the key advantage of gamma delta T cells is in their ability to identify antigens out of the context of classical major histocompatibility complex (MHC) molecules and natural tropism of γδT cells for the tumor microenvironment. [5], [6]

γδ T cells are a heterogeneous population of cells characterized by T cell receptors (TCRs) formed from γ and δ chains. $δ1^+$ T cells are predominately connected with a γ2/3/4/5/8 chain and reside in epithelial tissues. Majority of $δ2^+$ T cells are connected with γ9 chain and constitute the main pool of the peripheral blood lymphocytes. [7] Over 70% of γδ T cells are double negative cells [$CD4^-CD8^-$], the rest of the cells are single positive with predominance of $CD8^+CD4^-$, 30%, and < 1% $CD4^+CD8^-$. [3]

Many studies have shown that the amount of γδ T cells infiltrating solid tumors is a strong predictor of a favorable outcome for cancer patients. [8] Taking into account this fact and all attractive properties of γδ T cells, expectations for γδ T cell use in adoptive immunotherapy. Clinical trials conducted in numerous cancers confirmed the safety of γδ T cells. [9] Unfortunately, the efficacy of γδ T cell immunotherapy has been limited. This is hypothesized to be due to ambiguous effects of specific γδ T cell subsets on cancer cells. Furthermore, anergy or exhaustion of the effector γδ T cells has been observed after induction by intravenous n-aminobisphosphonates or phosphorylated antigens. Recent studies have also revealed that γδ T cells may promote carcinogenesis by inhibition of antitumor response, promoting cancer angiogenesis, invasion, and metastasis. [10] Thus it is unclear whether the role of these cells is in acute leukemia is to inhibit or promote tumor progression.

Heat-shock proteins (HSPs) are highly conserved molecules and have been extensively studied in the context of carcinogenesis, especially their extracellular form. HSPs in free form or HSP–peptide complexes have been found to elicit antitumor immunity by acting as tumor-specific antigens, and adjuvants that facilitate uptake, processing, and presentation. [11], [12]

According to many reports, γδ T cells demonstrate a strong association to HSPs by different mechanisms including direct recognition of specific epitopes or HSPs in free form or as peptide-HSPs complexes. [13]

The aim of our study was to evaluate the association between the number of γδ T cells and well-known prognostic factors in childhood ALL to verify the potential use of these lymphocytes as a novel biomarker of leukemia severity. Additionally, in our previous report we found decreased HSP90 serum level in patients at disease presentation and after induction block of the chemotherapy in comparison to healthy controls. [14] Due to the limited reports concerning the frequency of γδ T cells and their correlations with extracellular HSPs in cancers, including childhood ALL, we examined relationship between these two parameters in peripheral blood of 19 leukemic patients on the day of diagnosis and after induction therapy.

2. Methods

2.1. Patients

This study was approved by the local research Ethics Committee. All samples were obtained under the written informed consent. Nineteen patients (9 male and 10 female) aged 1 to 14 years were recruited into the study. Diagnosis of acute lymphoblastic B-cell leukemia was verified via cytological, cytochemical, and immunophenotyping methods. Blood samples were collected twice: before induction and on the the day when remission status was evaluated (33 day of therapy). Between these two time points, all patients received chemotherapy following protocol IA according to the ALL IC BFM 2009. In one of the patients, due to BCR/ABL mutation, along with standard treatment, imatinib was applied. At the end of the protocol, 13 of the patients were classified to intermediate risk (IR) group, 5 to high risk (HR), 1 patient was grouped as standard risk (SR). Clinical characteristics of study patients are shown in Table 1. Data regarding hematological parameters were obtained from medical records. Due to the small group of patients, we could not to analyze correlations between γδ T cells and cytogenetic, immunophenotypic, and central nervous system (CNS) status.

The control group consisted of 19 healthy people, aged 1-18 years (11 male and 8 female). Children with active infection, acute/chronic diseases, receiving immunosuppressive therapy or with oncological history were excluded.

Age	1–5 years = 16	6–11 years = 2	12–18 years = 1
Gender	Male = 9	Female = 10	
Risk group	SR: 1	IR: 13	HR: 5
CNS involvement	Positive: 3	Negative: 17	
Steroid sensitivity	Good: 18	Poor: 1	
BM on day 15	M1: 13	M2: 5	M3: 1
MRD	< 0,1% : 4	0,1 – 10% : 13	> 10% : 2
BM on day 33	M1: 19	M2: 0	M3: 0

Table 1: Patient characteristics.

2.2. Evaluation of serum HSP90

Blood samples from patients and healthy donors were allowed to clot, centrifuged and stored at -80°C for later analysis. Serum HSP90 concentrations were evaluated by enzyme-linked immunosorbent assay (ELISA) (Cloud Clone Corp., human serum HSP90 ELISA Kit) according to the manufacturer's instructions. The minimum detectable dose of HSP90 was described as less than 1.22ng/mL. The optical densities were determined by measuring the absorption at 450 nm. HSP levels are presented in units of ng/ml.

2.3. Evaluation of gamma-delta T cells

Freshly obtained EDTA whole blood was stained using antibody cocktails as follows: TCRγδ FITC (clone, IMMU510) / TCRαβ (clone, IP26A) / HLA-DR PC5 (clone, B8.12.2) / CD4 APC (clone, 13B8.2) / CD8 AF700 (clone, B9.11) / CD3 Krome Orange (clone, UCHT1) and CD45RA FITC (clone, 2H4LDH11LDB9) / CD31 PE (clone, 1F11) / TCRγδ PC5.5 (clone, IMMU510) / TCRγδ1 PC7 (clone, R9.12)/ CD4 APC (clone, 13B8.2) / CD8 AF700 (clone, B9.11) / CD62L AF750 (clone, DREG56) / TCRγδ2 Pacific Blue (clone, IMMU 389)/ CD3 Krome Orange(clone, UCHT1). Samples were then lysed with the Immunoprep Reagent Kit and TQPrep Workstation (Beckman Coulter, USA). Finally, fluorescence beads for absolute counting ware used. For the sample readout, Navios flow cytometer was used and data were analyzed with Kaluza Software (all from Beckman Coulter, USA). Data were interpreted according to fluorescence minus one approach.

2.4. Statistical analysis

Statistical analysis was performed using IBM SPSS 25. Depending on the distribution of variable, nonparametric Mann–Whitney U test, Wilcoxon test, or parametric Student's t-test were used to compare the differences between the normal control and leukemic patient group as well as differences among study group. The correlation between γδ, prognostic factors, and serum HSP90 in leukemic patients were analyzed by the correlation (R Spearman, Pearson). A value of $p < 0.05$ indicated statistical significance.

3. Results

3.1. Evaluation of γδ T-cells in peripheral blood at diagnosis and after induction chemotherapy among ALL patients versus controls.

We found that the level of activated CD3+ γδ T was increased in healthy controls in comparison to the ALL patients at diagnosis ($p = 0.02$). [Fig. 1] Additionaly, higher percentage of activated CD3+ γδ T cells were obseved in patients following chemotherapy compared to the subset before treatment ($p = 0.02$).

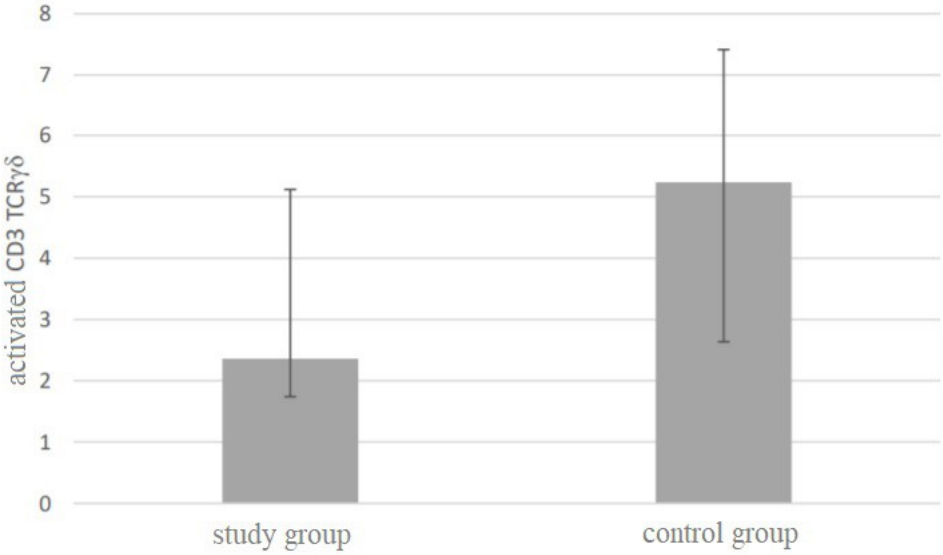

Fig. 1: Activated CD3 TCR γδ T cells in study group at diagnosis vs control group.

We showed no statistical differences in distribution of $CD3^{+}\gamma\delta$ ($p = 0.08$), $CD8^{+}$ γδ ($p = 0.92$), $CD4^{+}$ γδ ($p = 0.51$) in the study group vs healthy controls. There were also no differences in distribution of the subsets at diagnosis among children suffering from ALL before and after treatment $CD3^{+}\gamma\delta$ ($p = 0.87$), $CD8^{+}$ γδ ($p = 0.39$), $CD4^{+}$ γδ T cells ($p = 0.57$).

3.2. Evaluation of γδ T-cells among IR and HR group of patients.

Due to the only one patient classified to standard risk group, he was excluded from the evaluation. The amount of activated $CD3^{+}$ γδ T cells was higher following induction both in the IR and HR patients. However, statistical difference was noticed in the high risk (HR) group ($p = 0.04$), but not in the IR group ($p = 0.24$). [Fig.2]

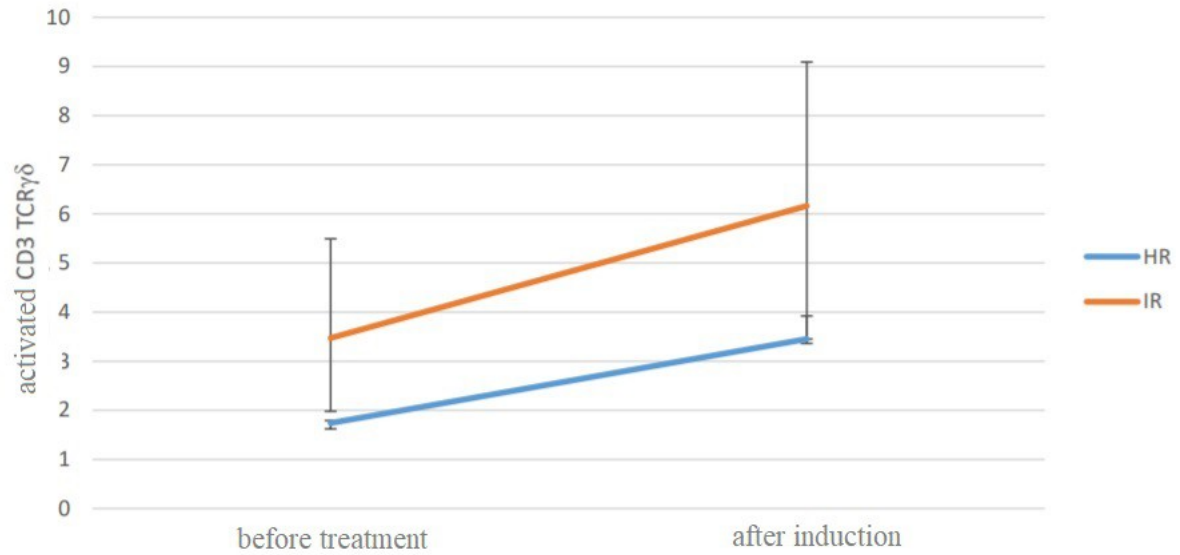

Fig. 2: Activated CD3 TCR γδ T cells before vs after treatment in the IR and HR patients.

The percentage of CD8+ γδ T cells was definitely higher in the IR group before treatment relative to the HR group at diagnosis (p = 0.03) [Fig. 3] and the tendency was maintained after induction chemotherapy (p = 0.02).

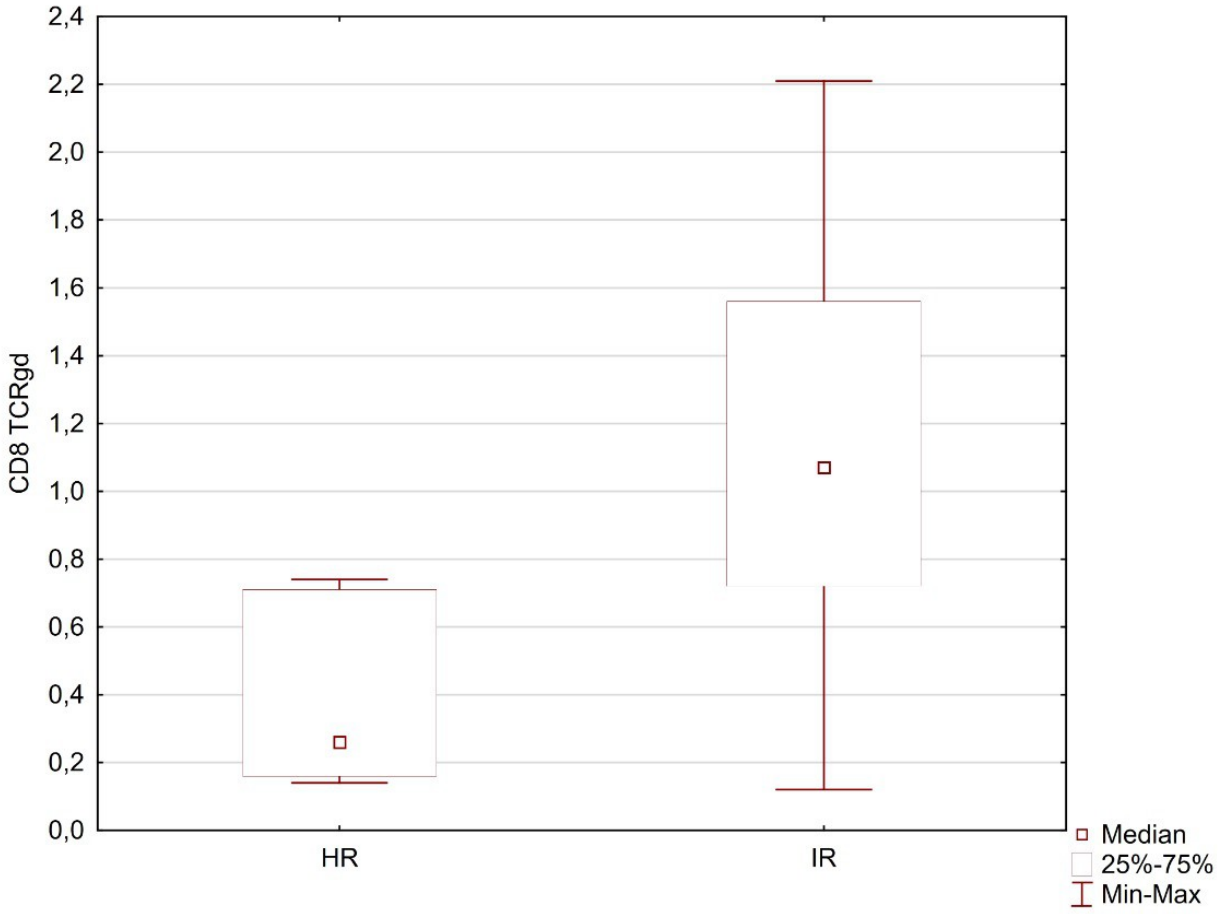

Fig. 3: CD8+ γδ T cells at diagnosis in the IR group vs HR group.

3.3. Correlations of γδ T cells and risk factors.

We evaluated the association of γδ T cells with a series of clinical and hematological agents. Interestingly, we observed differences the percentage of $CD8^{+}$ γδ depend on age in leukemic

patients at diagnosis (R = -0.67, p = 0.002) [Fig. 4] and on 33 day of therapy (R = -0.78, p < 0.001) [Fig. 5], but not in healthy controls (p = 0.58). The same investigation showed no statistically relevant differences the percentages of other subsets of lymphocytes among leukemic patients in two time points (before and after treatment) as well as the subsets vs healthy controls. [Table 2]

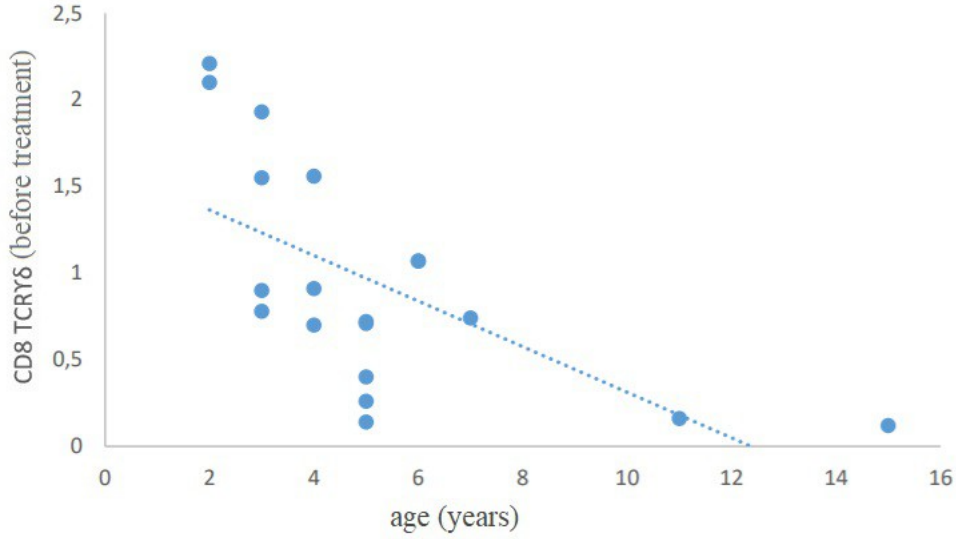

Figure 4: Correlation of CD8+ γδ Tcells with age among leukemic patients before treatment.

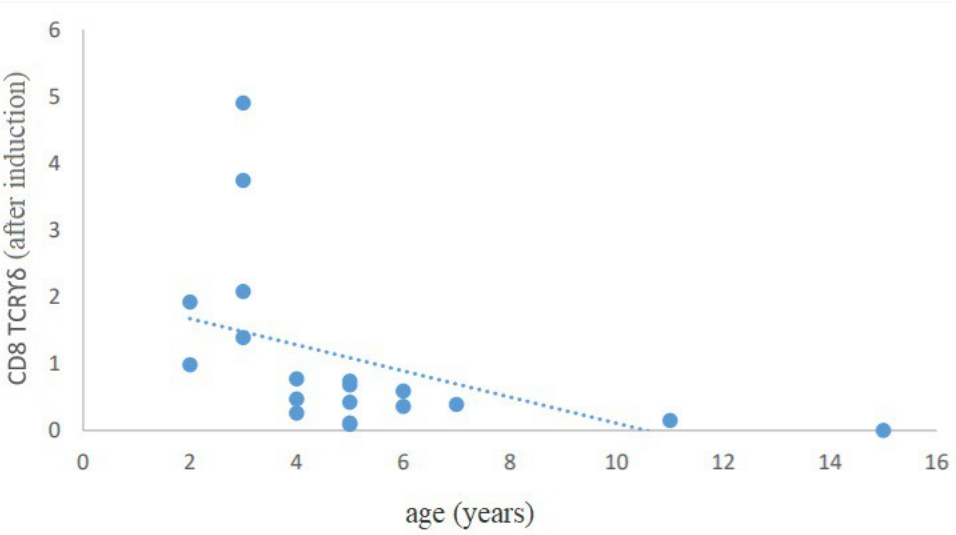

Figure 5: Correlation of CD8+ γδ Tcells with age among leukemic patients after treatment.

		Spearman	P-value
Study group - day 0	CD3+ γδ	-0,36	0,127
	CD3+ γδ act.	-0,06	0,804
	CD4+ γδ	-0,23	0,353
	CD8+ γδ	-0,67	0,002
Study group - day 33	CD3+ γδ	-0,42	0,071
	CD3+ γδ act.	-0,12	0,630
	CD4+ γδ	-0,43	0,067
	CD8+ γδ	-0,78	0,000
Control group	CD3+ γδ	-0,09	0,705
	CD3+ γδ act.	*	*
	CD4+ γδ	-0,10	0,669
	CD8+ γδ	-0,13	0,587

Table 2: Correlations of γδ T cells based on age in study group before and after chemotherapy vs healthy controls. * - Pearson 0,15 p = 0.53.

We did observe significant negative correlations between $CD8^+$ γδ T cells at presentation and after induction with two crucial prognostic factors in childhood ALL, minimal residual disease and lymphoblasts on the 33rd day of therapy. The $CD8^+$ γδ T cell correlation with MRD before chemotherapy was R = -0.53, $p < 0.05$ [Fig. 6] and after chemotherapy, R = -0.59, $p < 0,01$. [Fig. 7] Further, the $CD8^+$ γδ T cell correlation with lymphoblasts on the 33 day of therapy before induction was R = -0.44 and after induction was R = -0.38. However, probably due to the small group of patients, the correlations were not statistically relevant.

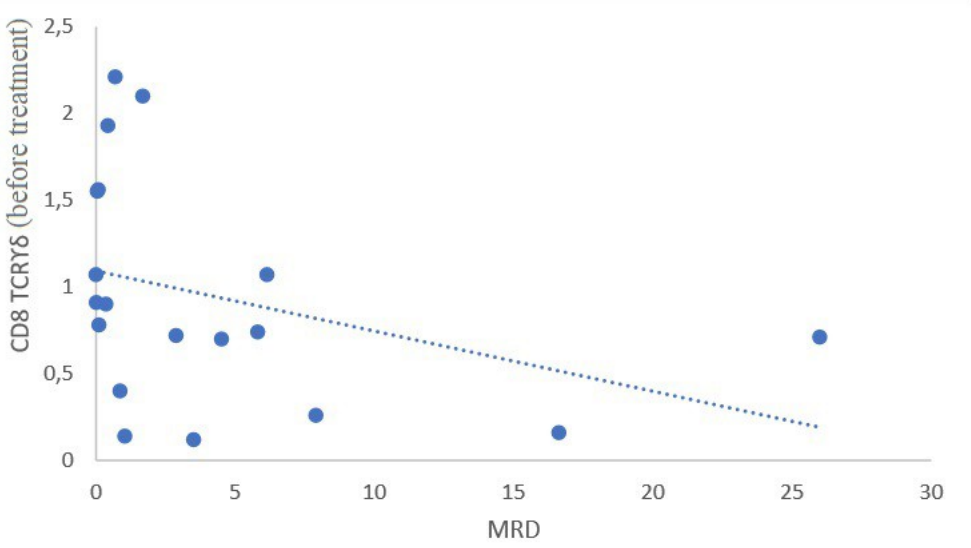

Fig. 6: Correlation of CD8+ γδ T cells at diagnosis and minimal residual disease (MRD).

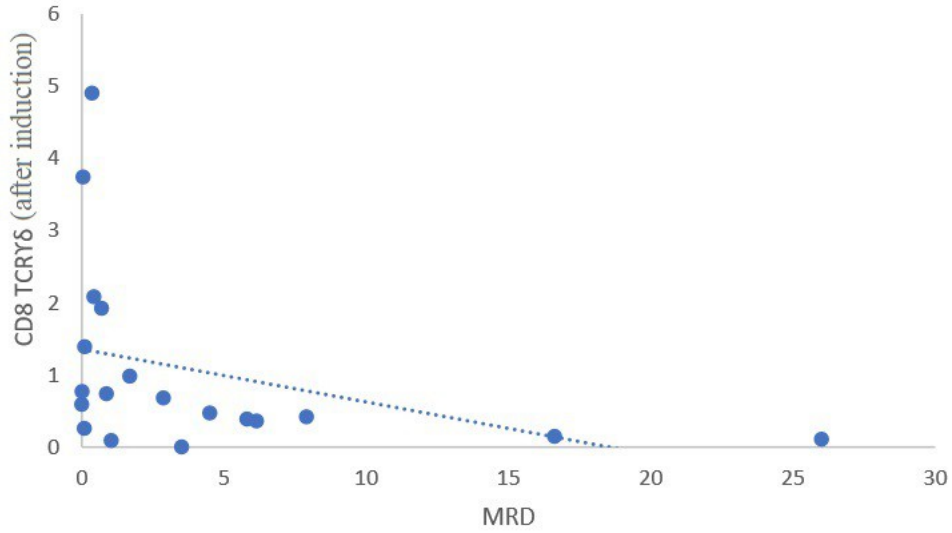

Fig. 7: Correlation of CD8+ γδ T cells after induction and minimal residual disease (MRD).

3.4. Comparison of γδ T cells and serum HSP90 with peripheral blood of ALL patients versus normal controls.

Analysis showed an association between activated CD3+ γδ T cells and serum HSP90 in ALL patients before treatment, R= 0.53, $p < 0.05$. [Fig. 8] We also found an association between CD8+ γδ T cells and serum HSP90, R= 0.31. The same investigation showed no statistically relevant differences among healthy controls respectively R= -0.2 and R = 0.12.

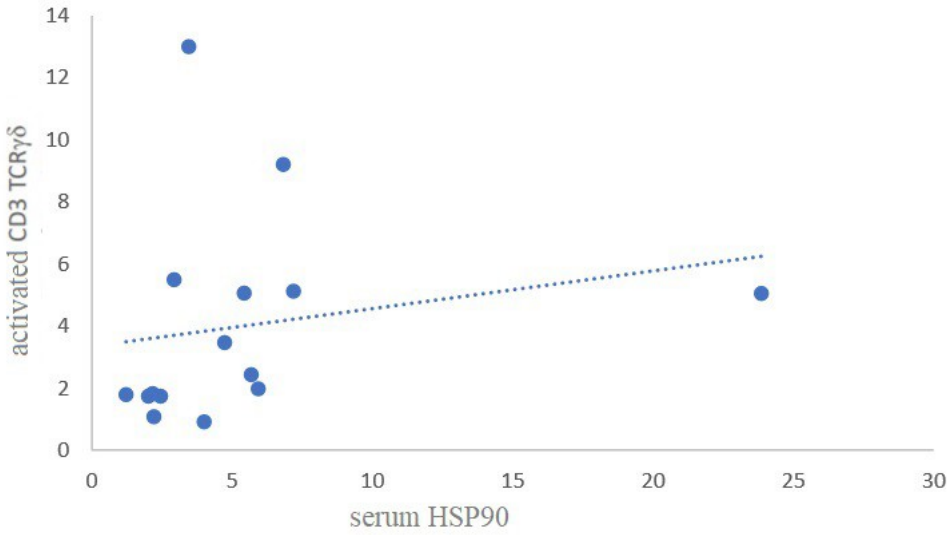

Fig. 8: Correlation of activated CD3+ γδ T cells and serum HSP90 among children with ALL at diagnosis.

4. Discussion

γδ T cells represent a subset of T cells in peripheral blood and due to their unique features, they are considered as good candidates for effective antitumor therapy. These cells are responsible for the production of cytotoxic cytokines, stimulation of macrophages and DCs, and recognition of antigens without the presence of MHC. [4]

Increased amounts of γδ T cells are observed in the tumor microenvironment of several types of solid cancers. In some cases, these cells are identified as the most significant favorable cancer-wide prognostic signature for outcome. [8] On the other hand, there are also reports demonstrating their pro-tumor role due to secretion of IL-17 and promotion of chronic inflammation. [20] A high percentage of γδ T cells in breast tumors was positively correlated with advanced tumor stages, HER2 expression, lymph node metastasis, and following poor outcome. It has been revealed that some Vδ1 populations of γδ T cells present immunosuppressive properties such as inhibition of DC maturation, suppression of T cell proliferation, and IL-2 secretion, which could potentially be responsible for failure of anti-cancer therapy. [15]

When it comes to hematological malignancies, γδ T cells seems to play clear an anti-tumor role. In chronic lymphocytic leukemia (CLL), circulating $V\delta1^+$ T cells are increased in up to 60% of patients [16] and have been associated with stable disease in low-risk CLL patients [17]. In adult acute myeloid leukemia (AML), the count of circulating γδ T cells was shown to be lower at diagnosis compared with healthy volunteers. Interestingly, lower counts of γδ T cells after chemotherapy was associated with more active disease in the same study. [18]

To our knowledge, no data has been reported to date on γδ T cell counts among pediatric patients with acute leukemia at diagnosis and during chemotherapy. Similarly, no evidence has been presented previously about lymphocytes and their correlation with already known risk factors in childhood ALL. Thus far, γδ T cell counts have been examined in children with ALL following hematopoietic stem cell transplantation (HSCT). The results of the study also argued in support of an anti-cancer role for γδ T cells. γδ T cells have been found with increased frequency in patients who proceed to become free of disease after HSCT compared to patients with normal or decreased numbers of γδ T cells. [19] Meeh, PF, et al, showed that the lymphocytes exhibit an in vitro response to primary leukemia blasts that is manifest by proliferation of $\delta1^+$ T cells, and increased cytotoxicity to the primary leukemia blasts. [20]

In our study we showed higher percentage of $CD8^+$ γδ T cells in younger patients. This correlation was rejected before and after induction chemotherapy, but not in the control group. Taking into account the fact, that explanation for the better outcome during the age of 1–6 and worse during adolescent is still unclear, it could be a good introduction to further research on lymphocytes involvement in age-related response to leukemia treatment. [21] Apart from it, we found strong negative correlation between $CD8^+$ γδ T cells and minimal residual disease (MRD), that is a key prognostic factor reflecting the number of lymphoblasts in bone marrow on 15 day of treatment. The amount of $CD8^+$ γδ T cells in peripheral blood was increased in those, who achieved the lower percentage of bone marrow blasts on 15 day of chemotherapy. This result is also supported by moderate negative correlations with number of lymphoblasts in bone marrow on day 33 of treatment - a post-induction marker of leukemia activity. Additionally, an interesting finding was definitely higher percentage of CD8+ γδ T cells in the IR group before treatment relative to the HR group at diagnosis. The tendency was maintained after induction.

All the results presented by our team suggest that particulair subset of γδ T cells - $CD8^+$ γδ T cells may play an important role in anti-leukemic response and may be a novel biomarker of favorable prognosis in childhood ALL. However, the findings require confirmation among a larger group of patients.

The association between activated CD3+ γδ T cells and serum HSP90 before treatment are promising for understanding the role of these cells in ALL. As mentioned, human γδ T cells can recognize and respond to a wide variety of stress-induced antigens including heat shock proteins, thereby developing broad anti-tumor and anti-infectious activity. [13] Our previous report revealed significantly decreased serum levels of HSP90 at diagnosis compared to healthy controls and higher concentrations of serum HSP90 after induction treatment. [14] In this report we showed similar results for the activated CD3+ γδ T cell subset and demonstrated statistically relevant correlations between both parameters at diagnosis, but not after induction chemotherapy. However, activated CD3+ γδ T cells, as well as serum HSP90, were increased after induction chemotherapy. Thus, it is suggested that serum HSP90 may play a key role in activation of the CD3+ γδ T cells in childhood ALL. The results are interesting due to the fact, that HSPs are currently under intensive investigation due to the autologous tumor-derived HSP peptide-based vaccines. [11] Therefore, there is preliminary evidence for the potentiating activation of γδ T cells with HSPs which could be an interesting direction of development of anti-cancer strategy for childhood ALL.

5. Conclusion

In summary, we have shown that high percentages of CD8+ γδ T cells may be a novel, early marker of favorable prognosis in childhood ALL. This may also indirectly indicate that γδ T cells play an important role in the anti-leukemic response. Strong correlations between activated CD3+ γδ T cells and serum HSP90 provide a promising suggestion that these cells may enhance the effect of conventional chemotherapy via supporting the immune system. Further studies are needed to determine clinical importance of our findings.

Abbreviations

γδ T cells: gamma-delta T cells
HSPs: heat shock proteins
ALL: acute lymphoblastic leukemia
CLL: chronic lymphoid leukemia
AML: acute myeloid leukemia
HSCT: hematopoietic stem cell transplantation
ALL IC BFM 2009: acute lymphoblastic leukemia intercontinental Berlin-Frankfurt-Munchen
NK cells: natural killer cells

DC: dendritic cells
SR: standard risk (group)
IR: intermediate risk (group)
HR: high risk (group)
CNS: central nervous system
MRD: minimal residual disease
PBMC: peripheral blood monoclonuclear cells
ELISA: enzyme-linked immunosorbent assay
PB: peripheral blood
BM: bone marrow
CD: cluster differentiation antigen
WBC: white blood cells

Data Availability
The statistical analysis (as Excel and Statistica files) used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

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