Am J Blood Res 2020;10(4):90-96
www.AJBlood.us /ISSN:2160-1992/AJBR0114780

*Original Article*

# Regulatory T cells in pediatric AML are associated with disease load and their serial assessment suggests role in leukemogenesis

Anuj Kumar Bansal[1,4,5], Surender Kumar Sharawat[1,4,5], Ritu Gupta[2,4], Sreenivas Vishnubhatla[3,5], Deepa Dhawan[1,4,5], Sameer Bakhshi[1,4,5]

*Departments of [1]Medical Oncology, [2]Laboratory Oncology, [3]Biostatistics, [4]Dr. B.R.A. Institute Rotary Cancer Hospital, [5]All India Institute of Medical Sciences, New Delhi-110029, India*

Received May 22, 2020; Accepted June 23, 2020; Epub August 25, 2020; Published August 30, 2020

**Abstract:** Background: Regulatory T cells (Tregs) modulate immune system by suppressing other immune cells. In current exploratory era of immunotherapy, the detailed enumeration data of Tregs cells in pediatric AML is lacking. Aim: Serial assessment of Treg absolute values in pediatric AML at diagnosis and follow-up; and correlating with outcome. Study design: Prospective study. Methods: Study objectives were determining Tregs (CD4+CD25+Foxp3+) were assessed at diagnosis, post-induction, post-consolidation, 3 and 6 months follow-up and relapse in 30 consecutive pediatric AML patients. Results: Patients with AML had higher baseline Treg frequencies than controls (P=0.0001). Female patients, WBC > 50,000 × $10^3$/L and hypoalbuminemia were significantly associated with high Treg absolute values. Baseline Tregs were not associated with DFS, EFS and OS. Tregs significantly decreased after induction chemotherapy (P=0.028). Using generalized-estimating-equation regression model, Treg absolute numbers continued to decrease at each assessment time point from post-induction till 6 months follow-up (P=0.029) in those who are in continuous CR; however, in those patients who relapsed, Tregs did not change from post-induction till last follow-up preceding relapse (P=0.39). Conclusions: This first study in pediatric AML demonstrates that Tregs are increased at diagnosis; the increased number is significantly associated with female gender and high WBC count. Tregs decrease after induction chemotherapy as compared to their baseline value. Post CR, Treg absolute values continue to decrease significantly in those who stay in CR but not so in those who relapse; this suggests their possible role in leukemogenesis.

**Keywords:** Acute myeloid leukemia, CD25, Foxp3, pediatric, Treg cells

## Introduction

Regulatory T cells (Tregs), a subgroup of CD4+ T cells, are characterized by positivity for CD4, CD25 and Foxp3 [1]. Studies suggest that Tregs may be responsible for the commencement and progression of acute and chronic leukemias [2-4]. The malignant cell suppresses immunity with increased immunosuppressive Treg cells in pediatric acute lymphoblasic leukemia, and after induction chemotehrapy their numbers decrease significantly [2]. Wan et al confirmed the role of Tregs in the immune escape of myeloblast in *de novo* acute myeloid leukemia (AML) [5].

Role of Tregs in adult patients with AML has been explored at baseline but data on their follow-up is limited [6]. There are no existing data on Tregs in pediatric patients with AML. The primary objectives of this study were to determine Treg absolute values in pediatric AML patients at diagnosis; correlate the same with baseline patient characteristics and to observe their change during course of therapy and on follow-up with treatment. The secondary objectives were to correlate Treg absolute values at baseline with outcome based on complete remission (CR) rate, event free survival (EFS), disease frees survival (DFS) and overall-survival (OS).

## Materials and methods

### *Patients, treatment and sampling*

From November 2010-May 2011, consecutive *de novo* patients with AML aged 1-18 years treated at our cancer hospital were prospectively enrolled. Patients with acute promyelocytic leukemia (APML), immunodeficiency disor-

der and documented infection at diagnosis were excluded. Institute ethics committee approved study and informed written consent was taken from guardian. Induction therapy consisted of Daunorubicin 60 mg/m$^2$/days for 3 days with Cytarabine 100 mg/m$^2$/day for 7 days; patients not in remission post first induction received cytarabine, daunorubicin and etoposide. Patients not in CR after 2 inductions were declared refractory. Post CR, patients received three cycles of cytarabine at 18 gm/m$^2$.

Peripheral blood (10 ml) at preselected time points was processed within 24 hours: at baseline (Treg-0), post first induction (Treg-1), post treatment completion (Treg-2), at 3 months (Treg-3) and 6 months (Treg-4) after treatment completion, and at relapse (Treg-R). Patients on follow-up were divided into two groups: group A included patients who maintained CR till last follow-up and group B included those who relapsed. Six age and sex matched healthy control blood samples were also collected.

*Flowcytometry*

Mononuclear cells (MNC) were separated from peripheral blood using density gradient media (Ficoll hypaque; Sigma 1077, Sigma-Aldrich, Inc. USA). Isolated MNCs were resuspended in phosphate-buffered-saline. One million peripheral blood MNCs were incubated with CD4 phycoerythrin cyanine-5 and CD25 fluorescein isothiocyanate (BD Biosciences, USA) antibodies for surface staining followed by fixation and permeabilization for Foxp3 phycoerythrin (BD Biosciences, USA) staining as per manufacturer's recommendations. CD4+ cells were gated from total lymphocytes and subsequently on CD4+ cells, Foxp3 and CD25 dual positive cells were characterized as Tregs. Total $1 \times 10^5$ events were acquired on flowcytometer (FACS Caliber, BD Biosciences, USA) and analysis done using CellQuest Pro software. Unstained MNCs were acquired to negate auto-fluorescence. Treg frequency (perecntage) was the positive cells from total CD4+ cells of gated lymphocytes in lymphoprep enriched cells, while absolute numbers of Tregs per cubic millimeter were calculated from absolute lymphocyte counts.

*Statistical analysis*

EFS was defined as time between diagnosis and first event such as failure to achieve CR, relapse or death; DFS as time from CR until relapse; and OS as time between diagnosis and death. Survival time was censored at last follow-up on October 31, 2018. Data were expressed as mean ± SD and differences between values were determined using student's t test. Associations of Tregs and patients baseline characteristics were analyzed by Fisher's exact test/Chi square test; Pearson correlation was done for continuous variables. Generalized estimating equation (GEE) regression model was used to evaluate trend of Tregs in group A and B patients on follow-up. Kaplan Meier curve were obtained for survival analysis followed by log rank test. *P* value was log transformed and value of $\leq 0.05$ was considered significant. All statistical analysis was done with stata statistical software version 11 (StataCorp LP, Texas, USA).

## Results

*Patients and outcome*

During study period, 34 patients with *de novo* AML were registered. Four patients were excluded (1 APML, 1 secondary AML, 1 patient was < 1 year and another had pneumonia). Out of remaining 30 consecutive eligible patients, the median age was 9.5 years (range 1-18 years). There were 6 controls with median age 7 years (range: 4-14 years). The mean age of controls was not significantly different from that of patients (10.4±4.62 versus 7.5±3.4, P=0.15).

Of the 30 patients, 25 (83.3%) achieved CR post-induction (2 septic deaths; 2 refractory to chemotherapy, 1 defaulted during induction). All 30 patients were available for EFS and OS, and 25 CR patients were evaluable for DFS. For the entire cohort of 30 patients, the median follow-up time was 17.7 months (0.5-97.8). At five years, DFS, EFS and OS was 43.6±9.9% (24.1-61.7), 36.3±8.8% (19.7-53.1) & 42.5± 9.1% (24.6-59.3) respectively.

*Baseline Treg frequency and correlation with patient characteristics*

Median baseline Treg frequency in patient cohort was 11.66%. Baseline mean Treg frequency in pediatric patients with AML and healthy controls were 12.38±4.65% (range 6.2-26.6%) and 3.16±1.49% (range 1.65-4.86%)

**Table 1.** Correlation of Treg frequency and absolute numbers with baseline patient characteristics

| | Treg % | *P* value | Treg Absolute Numbers | *P* Value |
|---|---|---|---|---|
| Mean ± SD | 12.35±4.65 | | 1507.3±2789 | |
| Sex | | | | |
| Male (14) | 10.5±2.5 | | 443.3±587 | 0.0152 |
| Female (16) | 14±5.5 | 0.044 | 2371.8±3530.4 | |
| Age | | | | |
| ≤ 9 yr (15) | 12.8±5.6 | | 1205.7±2676.4 | 0.2835 |
| > 9 yr (15) | 11.8±3.6 | 0.70 | 1830.4±2969.8 | |
| Fever | | | | |
| Yes (28) | 12.0±4.2 | | 1560.7±2879.1 | 0.7149 |
| No (2) | 17.1±10 | 0.22 | 786±1063.4 | |
| Albumin (g/dl) | | | | |
| > 3.5 (23) | 10.7±2.1 | | 600.8±677.4 | 0.0234 |
| ≤ 3.5 (7) | 17.6±6.7 | 0.002 | 4356.2±4712.4 | |
| GS | | | | |
| Yes (8) | 12.3±5.4 | | 488.9±614.6 | 0.3363 |
| No (22) | 12.4±4.5 | 0.85 | 1895.2±3191.1 | |
| CSF | | | | |
| Positive (4) | 12.2±2.2 | | 728.8±666 | 0.7094 |
| Negative (25) | 12.5±4.9 | 0.88 | 1630.8±3047.6 | |
| NA (1) | | | | |
| LDH (U/l) | | | | |
| < 300 (2) | 16.3±5.9 | | 3964.7±4620.5 | 0.3425 |
| 301-1000 (12) | 10.7±2.9 | | 1172.5±3013.4 | |
| > 1000 (11) | 10.9±2.67 | 0.15 | 1609.6±2849.2 | |
| NA (5) | | | | |
| Hemoglobin (g/dl) | | | 1628±3114.9 | 0.4120 |
| ≤ 7 (15) | 11.1±3.6 | | 1378±2503.3 | |
| > 7 (15) | 13.6±5.6 | 0.14 | | |
| Platelets × $10^9$ | | | | |
| ≤ 20 (11) | 11.7±5.8 | | 1579.6±3075.1 | 0.9164 |
| > 20 (19) | 12.7±3.9 | 0.30 | 1463.1±2691.1 | |
| WBC × $10^9$ | | | | |
| ≤ 50 (23) | 11.3±3.9 | | 414.1±460.3 | 0.0001 |
| > 50 (7) | 15.8±5.4 | 0.023 | 4943±4171.2 | |
| FAB Subtype | | | | |
| M2 (19) | 12±4 | | 540.3±668.4 | |
| M4/5 (3) | 11.1±4.1 | 0.20 | 4118.4±5713.2 | 0.750 |
| Others (8) | 17.1±6.2 | | 2284±2801.5 | |
| Cytogenetics | | | | |
| Favorable (13) | 13.3±6 | | 1402.8±2956.7 | 1.3704 |
| Intermediate (12) | 12.2±3.4 | | 1944.7±3186.3 | |
| Poor (3) | 11.4±2.4 | 0.92 | 355±413.5 | |
| Indeterminate (2) | | | | |

SD: standard deviation, GS: granulocytic sarcoma, CSF: cerebrospinal fluid, NA: not available; FAB: French American British.

respectively (P < 0.001). In our cohort, Treg frequency and absolute numbers were significantly higher in female geneder, those with WBC > 50,000 × $10^3$/L and hypoalbuminemia (**Table 1**).

*Trend of Treg absolute numbers during therapy and on follow-up*

There was significant decrease in Treg absolute numbers after induction chemotherapy (P=0.028) (**Figure 1A**). At relapse, Treg absolute numbers were not different as compared to the pre-relapse Treg absolute numbers (P=0.136) (**Figure 1B**).

Using GEE regression model, in group A patients from post-induction till 6 months follow-up, Treg absolute numbers decreased (20/assessment, P= 0.029); however, in group B patients from post-induction till last follow-up preceding relapse, Treg absolute numbers decreased but this reduction was not significant (7/assessment, P=0.39) (**Figure 2**). At each pre-selected time point, there was no significant difference in Treg absolute numbers between group A and group B patients (**Table 2**).

*Correlation of Tregs with outcome*

Mean baseline Treg absolute numbers in patients who achieved CR (n=25) versus those who did not achieve CR (n=5) was 1374.2±2802 and 2145.9±2946.2 (P=0.58). DFS, EFS and OS in patients with higher Treg absolute number (above median) when compared with lower Treg absolute number (below median) was

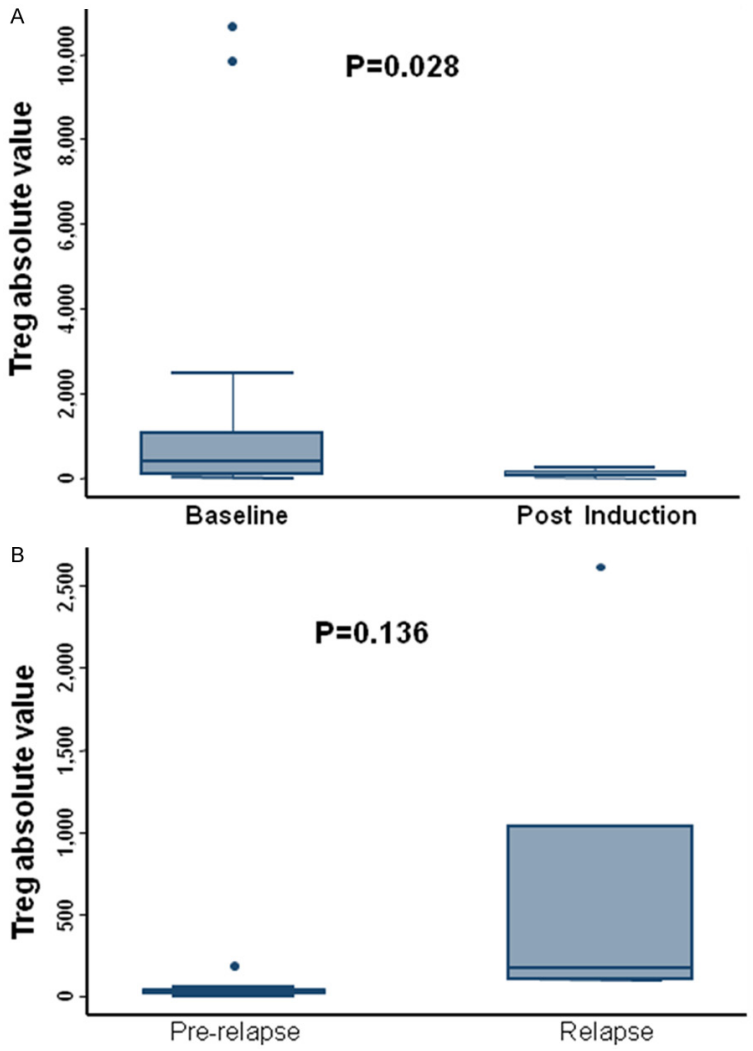


**Figure 1.** Significant decrease of Treg absolute values from baseline to post induction in patients who achieve complete remission using paired t test (A). Treg absolute values at relapse when compared to the pre-relapse value in group B patients using paired t test (B).

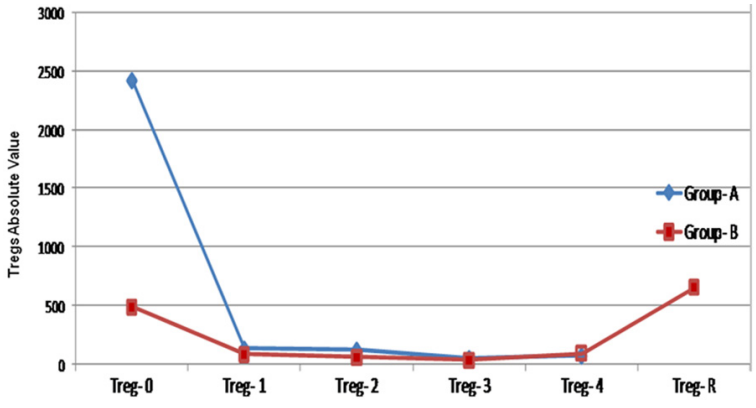


**Figure 2.** Serial mean values of absolute Treg values in group-A and group-B patients (N=25, that is patients who achieved CR post induction); graph shows significant decrease of Treg absolute values in group A patients (P=0.029) but not so in group B patients (P=0.39) during follow up.

not significantly different [38± 13% (13-62) Vs 50±14% (23-78), HR 0.68 CI (0.23-1.96) P=0.473; 40.0±12.7% (16.5-62.8) Vs 33.3±12.2% (12.1-56.4), HR 0.89 CI 0.4-2.2, P=0.80; 40.0±12.6% (16.5-62.7) Vs 45.7±13.1% (20.1-68.3) HR 1.07 CI (0.41-2.78) P=0.889] (**Figure 3A-C**).

## Discussion

In this prospective study, we demonstrated that pediatric patients with AML have higher Treg frequency at diagnosis as compared to normal healthy controls. Data in adult AML also suggests that Tregs are higher in patients with AML than healthy controls [7, 8]; however, only two previous studies had used Foxp3 for Treg characterization [4, 8]. Other studies in adult AML analyzed Tregs as a subset of CD4+ cell population using $CD25^{high}$ or $CD127^{low}$ for characterization [9, 10]. The paired analysis of Tregs at diagnosis and with treatment after Midostaurin (FLT3 inhibitor) demonstrated a significant decrease in CD4+CD25+ Foxp3+ T cell population and Foxp3 mRNA expression in AML PBMCs as well as in healthy individuals [11]. Similarly, Tregs (CD4+CD25+ cells) were high in adult patients with AML as compared to their level post remission and in those who were healthy adult controls [12]. CD25 is known not to be specific for Tregs, as it is also expressed by effector T-cells. Our study is the first of its kind wherein Tregs were assessed at diagnosis and on serial follow up in consecutive pediatric AML patients prospectively using CD4, CD25 and Foxp3 for characterization. Notably, we did not have

**Table 2.** Absolute Treg cells at diagnosis, post-induction, post-consolidation, 3 and 6 months follow-up and relapse

| | Treg-0 Mean ± SD (n) | Treg-1 Mean ± SD (n) | Treg-2 Mean ± SD (n) | Treg-3 Mean ± SD (n) | Treg-4 Mean ± SD (n) | Treg-R Mean ± SD (n) |
|---|---|---|---|---|---|---|
| Whole group (n=25) | 1374.2±2802 (24) | 101.2±77.2 (25) | 85±98.4 (22) | 39.1±32 (14) | 69±54.5 (10) | 651±926.1 (7) |
| Group-A | 2424±3943 (11) | 122±91 (12) | 113.1±120.2 (11) | 41.2±34.4 (10) | 64±58 (8) | - |
| Group-B | 486±478 (13) | 82.2±60 (13) | 57±64.4 (11) | 34±28 (4) | 88±39.4 (2) | 651±926.1 (7) |
| * *p* value | 0.2073 | 0.2273 | 0.2758 | 0.6501 | 0.3637 | NA |

* *p* value comparing group-A with group-B using unpaired t test.

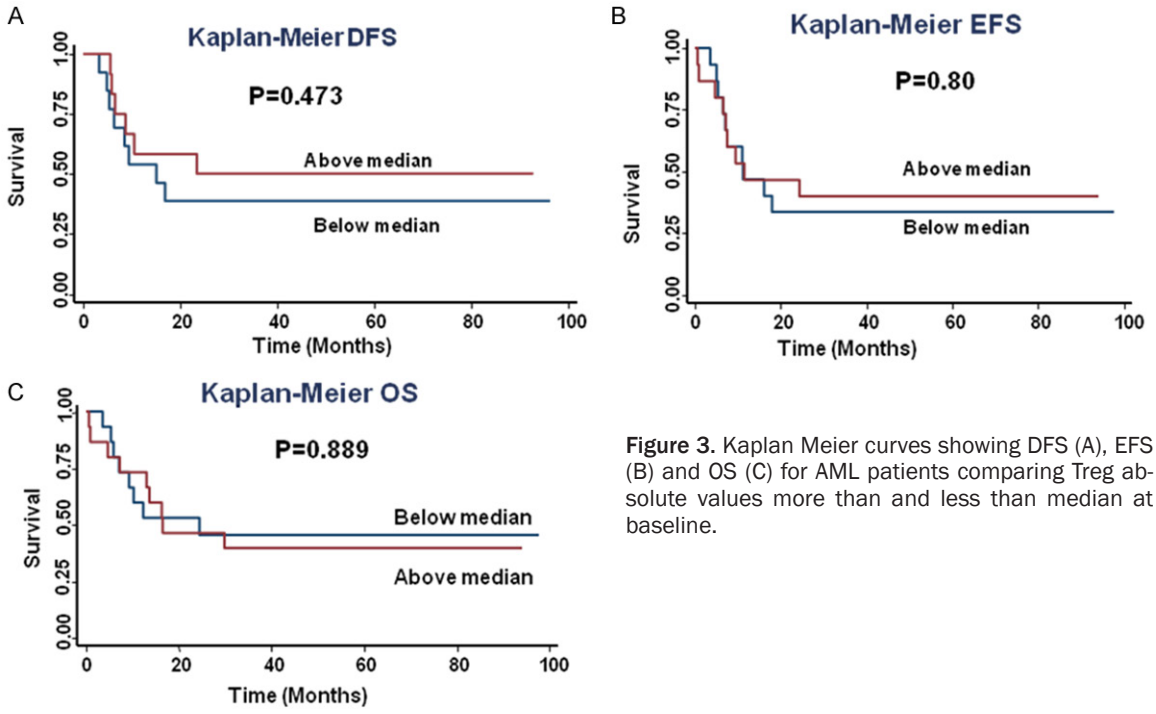


**Figure 3.** Kaplan Meier curves showing DFS (A), EFS (B) and OS (C) for AML patients comparing Treg absolute values more than and less than median at baseline.

patients with infections as control subjects, but previous data suggests that those with infection have higher Tregs as compared to healthy controls [13].

In our cohort, Treg frequency and absolute values were significantly more in patients with AML belonging to female gender as compared to males. In contrast, healthy children show higher Treg frequency in males as compared to females [14]. In solid tumors, no such difference has been demonstrated and data on Treg frequency in association with baseline demographic features in adult AML is not available. Our results are in accordance with previous hypothesis of presence of estrogen response elements in promoter regions of several immune genes [15]. In our study Tregs were significantly higher in patients with high WBC counts reflecting recruitment of Treg in circulation, which is directly related to change in microenvironment created by higher disease burden. Previously, in multiple myeloma and gastric cancer, it has been shown that Tregs increase with disease load [16, 17]. We also demonstrated higher Tregs in patients with hypoalbuminemia; however, we did not detect any significant difference in Tregs between the patients who have poor risk cytogenetic and those with good or intermediate risk karyotype.

Our finding of reduction in Tregs after induction chemotherapy in patients with AML is similar to a previous study in acute lymphoblastic leukemia wherein Tregs were high at baseline and decreased significantly after induction treatment [2].

Treg absolute values at baseline did not correlate with CR rates, EFS, DFS or OS; however, this study was not adequately powered for sur-

vival analysis, which was the secondary objective. Amongst patients who achieved a CR, there was a significant decrease in Tregs after induction chemotherapy. Our results are similar to studies in adult AML by Ersvaer et al (6 patients) [8] and Shenghui et al (32 patients) [9] while in contrast to Szczepanski et al (7 patients) who showed rise in Treg frequency post induction [10]. Xiang *et al* also demonstrated that Tregs were high in adult patients with AML and that they decreased after treatment [7]. Arandi *et al* demonstrated that increased Treg population was positively associated with immunosuppressive enzymes such as indoleamine 2,3-dioxygenase in cytogenetically normal AML which may be responsible for circumventing immune surveillance [18].

Interestingly, when serial follow-up data with respect to absolute Treg values was analyzed, we observed that patients who continued to stay in CR had a significant reduction in Treg while those patients who ultimately relapsed did not show a significant reduction in their absolute Treg values on serial follow-up after achieving CR; this suggests a possible role of Tregs in leukemogenesis. Although we had not measured minimal residual disease in our patient cohort, we postulate that patients who relapse are likely to have minimal residual disease which precludes a significant drop in Tregs on follow-up; on the other hand patients who are in continuous CR probably may not have minimal residual disease and thus, the drop in absolute Treg values is more pronounced.

To conclude, the major strength of this study is that this is the first studies of its kind which prospectively evaluated Tregs in sequential pediatric AML patients at baseline and on follow-up. This study showed that Tregs are significantly increased in pediatric patients with AML at diagnosis. Increased Tregs are associated with female gender and increase disease load. However, Tregs at baseline do not predict outcome. Tregs decrease post induction as compared to their baseline value. After achieving CR, on serial follow-up, Treg absolute values continued to decrease significantly in those who stay in CR but not so in those who relapse which suggests their possible role in leukemogenesis. The exact mechanism by which Tregs exercise this effect is largely unknown. Since Tregs act through cell to cell contact as well as secretion of cytokines, evaluation of their function and cytokine mileu may enhance our understanding of the role of Tregs in AML.

## Acknowledgements

Institute Research Grant, All India Institute of Medical Sciences, New Delhi-110029, India.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Sameer Bakhshi, Department of Medical Oncology, Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi-110029, India. Tel: 91-11-2658-8153; Fax: 91-11-26588663; E-mail: sambakh@hotmail.com

## References

[1] Gupta R, Ganeshan P, Hakim M, Verma R, Sharma A and Kumar L. Significantly reduced regulatory T cell population in patients with untreated multiple myeloma. Leuk Res 2011; 35: 874-8.

[2] Salem ML, El-Shanshory MR, Abdou SH, Attia MS, Sobhy SM, Zidan MF and Zidan AA. Chemotherapy alters the increased numbers of myeloid-derived suppressor and regulatory T cells in children with acute lymphoblastic leukemia. Immunopharmacol Immunotoxicol 2018; 40: 158-167.

[3] Bachireddy P, Burkhardt UE, Rajasagi M and Wu CJ. Haematological malignancies: at the forefront of immunotherapeutic innovation. Nat Rev Cancer 2015; 15: 201-15.

[4] Moon HW, Kim BH, Park CM, Hur M, Yun YM, Kim SY and Lee MH. CD4+CD25highFoxp3+ regulatory T-cells in hematologic diseases. Korean J Lab Med 2011; 31: 231-7.

[5] Wan Y, Zhang C, Xu Y, Wang M, Rao Q, Xing H, Tian Z, Tang K, Mi Y, Wang Y and Wang J. Hyperfunction of CD4 CD25 regulatory T cells in de novo acute myeloid leukemia. BMC Cancer 2020; 20: 472.

[6] Niedźwiecki M, Budziło O, Adamkiewicz-Drożyńska E, Pawlik-Gwozdecka D, Zieliński M, Maciejka-Kembłowska L, Szczepański T and Trzonkowski P. CD4+ CD25 high CD127 low/-Foxp3+ regulatory T-cell population in acute leukemias: a review of the literature. J Immunol Res 2019; 2019: 2816498.

[7] Xiang M, Guo L, Ma Y and Li Y. Expression of Th17 and CD4+ CD25+ T regulatory cells in peripheral blood of acute leukemia patients and their prognostic significance. Pak J Pharm Sci 2017; 30: 619-624.

[8] Ersvaer E, Liseth K, Skavland J, Gjertsen BT and Bruserud Ø. Intensive chemotherapy for acute myeloid leukemia differentially affects circulating TC1, TH1, TH17 and TREG cells. BMC Immunol 2010; 11: 38.

[9] Shenghui Z, Yixiang H, Jianbo W, Kang Y, Laixi B, Yan Z and Xi X. Elevated frequencies of $CD4^+$ $CD25^+$ CD127lo regulatory T cells is associated to poor prognosis in patients with acute myeloid leukemia. Int J cancer 2011; 129: 1373-81.

[10] Szczepanski MJ, Szajnik M, Czystowska M, Mandapathil M, Strauss L, Welsh A, Foon KA, Whiteside TL and Boyiadzis M. Increased frequency and suppression by regulatory T cells in patients with acute myelogenous leukemia. Clin Cancer Res 2009; 15: 3325-3332.

[11] Gutierrez L, Jang M, Zhang T, Akhtari M and Alachkar H. Midostaurin reduces regulatory T cells markers in acute myeloid leukemia. Sci Rep 2018; 8: 17544.

[12] Wang M, Zhang C, Tian T, Zhang T, Wang R, Han F, Zhong C, Hua M and Ma D. Increased regulatory T cells in peripheral blood of acute myeloid leukemia patients rely on tumor necrosis factor (TNF)-α-TNF receptor-2 pathway. Front Immunol 2018; 9: 1274.

[13] Whittaker E, Nicol M, Zar HJ and Kampmann B. Regulatory T cells and pro-inflammatory responses predominate in children with tuberculosis. Front Immunol 2017; 8: 448.

[14] Wang Y, Zhou L, Gong J, Zhou M, Shen F, Zheng X, Zhang W, Zhuang M, Lu W, Zhong P, Pan Q and Kang L. Frequency of the CD4+CD25nt/hiCD127lo regulatory T lymphocytes from the peripheral blood in Chinese healthy individuals. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2007; 23: 816-8.

[15] Polanczyk MJ, Carson BD, Subramanian S, Afentoulis M, Vandenbark AA, Ziegler SF and Offner H. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. J Immunol 2004; 173: 2227-30.

[16] Feyler S, von Lilienfeld-Toal M, Jarmin S, Marles L, Rawstron A, Ashcroft AJ, Owen RG, Selby PJ and Cook G. CD4(+)CD25(+)Foxp3(+) regulatory T cells are increased whilst CD3(+) CD4(-)CD8(-)alphabetaTCR(+) Double Negative T cells are decreased in the peripheral blood of patients with multiple myeloma which correlates with disease burden. Br J Haematol 2009; 144: 686-95.

[17] Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H and Fujii H. Localisation pattern of Foxp3+ regulatory T cells is associated with clinical behaviour in gastric cancer. Br J Cancer 2008; 98: 148-53.

[18] Arandi N, Ramzi M, Safaei F and Monabati A. Overexpression of indoleamine 2,3-dioxygenase correlates with regulatory T cell phenotype in acute myeloid leukemia patients with normal karyotype. Blood Res 2018; 53: 294-298.