

## Original Research Article

# Diversity of regulatory T cells in various immuno-physiological conditions

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### ABSTRACT

**Background:** Regulatory T cells (Tregs) play an active role in abrogating immune responses during microbial infections and are thus essential to maintain immune homeostasis and self-tolerance. Aberrant expression of Tregs can lead to spectrum of manifestations from allergy, chronic infections to immunopathological conditions such as autoimmunity and cancer. It is critical to have a clear understanding about the Tregs biology before attempting any possible interventions in humans. This study was undertaken to analyze the differential expression of Tregs in various physiological conditions.

**Methods:** Peripheral blood mononuclear cells (PBMCs) were extracted from normal human subjects (n=30), pregnant females (n=10), cord blood (n=10) and patients with various immunopathological conditions such as cancer (newly diagnosed non-Hodgkin's lymphoma, n=10), autoimmune conditions such as (type 1 diabetes mellitus-T1DM, Hashimoto's disorder, and other autoimmune disorders, n=10 each) and in donor grafts (n=10) following allogeneic hematopoietic stem cell transplant. Flow cytometry was performed to detect CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>Tregs and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup>Tregs.

**Results:** The levels of Tregs was found to be were significantly higher in cord blood from newborn infants, patients with T1DM and Hashimoto's disorder. In addition, the difference in Tregs level in pregnant females, patients with stem cell transplants, non-Hodgkin's lymphoma and other autoimmune disorders were not significant.

**Conclusions:** Till date, no comparative study was available about the status of Tregs population in normal and different physiological conditions from India. This is the first preliminary quantitative analysis of association of Tregs with different disorders. A clear cognizance of Tregs biology among various physiological conditions will be vital for targeting of these cells for any possible therapeutic modalities.

**Keywords:** Immuno-physiological, Cord blood, Autoimmunity, Cancer, T1DM, Pregnancy

### INTRODUCTION

The immune system of human beings has highly evolved to elicit a defensive response against a universe of pathogenic microorganisms. Also, unwanted immune responses to antigens as well pathogens can ultimately result in varied kinds of immuno-pathological conditions such as autoimmunity, cancer, metabolic complications and inflammatory diseases. Regulatory T cells (Tregs) are specialized subsets of cluster of differentiation 4 (CD4<sup>+</sup>)

cells which play an indispensable role in suppression of immune system by induction of tolerance.<sup>1</sup> Tregs regulate the tone and tenor of immune system by avoiding autoimmunity and several other pathological conditions.

There has been plethora of reports showing increased T suppressor cells activity may be associated with poor immune responses to tumor antigens and contribute to immune dysfunction.<sup>2,3</sup> It was demonstrated that these suppressor cells could be CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells.

Forkhead box P3 (FOXP3), a helix transcription factor was found to be critical for the development and control of Tregs. It has been used recently as a biomarker and a prognostic factor for malignant human tumors. Tregs are a recently discovered subset of T-lymphocytes with potent suppressive activity and pivotal roles in curtailing destructive immune responses and preventing autoimmune disease.<sup>4</sup>

Regulatory T cells (Treg) are essential to maintain immune homeostasis and self-tolerance. They do play an active role in suppressing immune responses during bacterial, parasitic and viral infections.<sup>5,6</sup> Whereas maximum expression of Tregs can result in deadly diseases such as cancer and other chronic infection, less of Tregs activity results in immunopathological conditions such as autoimmunity, and thus affects the pathogen dependent responses.<sup>7,8</sup> The discovery of regulatory T lymphocytes that are basically involved in maintenance of immune tolerance has shed some light on to the aspects of tolerance breakdown in different disease conditions.<sup>9,10</sup>

The therapeutic implications of Tregs in acute graft versus host diseases following allogenic hematopoietic transplantations have already been well documented.<sup>11</sup> Tregs have been also been known to play pivotal role in pregnancy.<sup>12,13</sup> CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> regulatory T cells (or CD127 dim regulatory T cells) express intracellular transcription factor FOXP3 which is vital for the maintenance of immune cell homeostasis and peripheral tolerance.<sup>14,15</sup> The critical role of Tregs has also been established in the regulation of innate immunity.<sup>16,17</sup> To our knowledge, there are no comparative studies investigating the levels of Tregs in different immunophysiological conditions.

The aim of this study was to evaluate the differential expression of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> or CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup>). This research will shed some light towards a better understanding of Tregs in various disease conditions.

## METHODS

### *Subject selection*

The study was conducted after obtaining blood samples in total of 100 subjects attending medical outpatient department (OPD) and inpatient department (IPD) at Jaslok Hospital and Research Centre, Pedder Road, Mumbai. The age of participants ranged between 0 months – 68 years across both sexes.

Prior to participation in the study, research approval from the ethical clearance committee of the hospital and a written informed consent was obtained from each subject. The indoor-patients, outdoor-patients of the Jaslok hospital as well as those referred from other hospitals were included in the study. Subjects were carefully selected on basis of selected inclusion and exclusion criteria.

### *Inclusion criteria*

The following inclusion criteria were strictly considered for selecting candidates for the study: normal healthy individuals of either gender, cord blood samples from new born infants, pregnant women (4-14 weeks), subjects with Hashimoto's disorder and newly diagnosed non-Hodgkin's lymphoma, subjects with autoimmune disorders, subjects with type 1 diabetes mellitus history and subjects undergone post allogenic stem cell transplant after engraftment.

### *Exclusion criteria*

The following exclusion criteria were strictly considered for selecting candidates for the study: any symptomatic or documented active infection, subjects receiving immunosuppressant including steroids in the past three months and peripheral blood eosinophilia.

### *Flow cytometric analysis*

#### *Sample preparation*

10 ml of peripheral blood was drawn from subjects. 2 ml of Ficoll-Hypaque (sigma aldrich) was over layered with 2 ml of 1:2 diluted blood samples. It was centrifuged at 2500 rpm in a swinging rotor centrifuge to obtain a buffy layer of PBMCs by density gradient centrifugation. The cells were carefully aspirated and washed twice with 1X phosphate-buffered saline (PBS) (sigma aldrich). The resulting pellet was resuspended in 1X PBS.

#### *Cell surface and intracellular staining*

Tregs were detected using the Tregs detection kit (130-094-158, Miltenyi Biotech, Germany) as per the manufacturer's instruction. Approximately 1×10<sup>6</sup> PBMCs were stained and incubated for 10 minutes in dark with 10 µl of CD4-FITC and CD25 antibodies (Miltneyi Biotech, Germany). Staining for CD127, a cell surface marker was performed using CD127 antibodies, human (clone: MB15-18c9 Miltneyi Biotech, Germany). Cells were washed with 1-2 ml of buffer per 10<sup>6</sup> cells at 300xg for 5 minutes at 4 °C followed by complete aspiration of supernatant. Upon fixation and permeabilization with fragment crystallizable receptor (FcR) blocking reagent, cells were stained intracellularly with 10 µl of the anti-FOXP3 antibody. Cells were then washed, resuspended in suitable amount of buffer and immediately utilized for flow cytometry analysis.

#### *Flow cytometric analysis*

Flow cytometry was performed on fluorescence-activated cell sorting (FACS) (BD Biosciences). Appropriate fluorochrome labelled, isotype-matched monoclonal antibodies (mAbs) were used as controls. Appropriate gating strategies were orchestrated to obtain correct

percentages of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> populations.

**Statistical analysis**

All statistical analysis was performed using GraphPad prism (GraphPad software, Inc., CA, US). First, data were tested for normal distribution by Kolmogorov-Smirnov test (KS) test. In case of normal distribution of data, statistical analysis was performed by unpaired and two-tailed student's t test (two independent groups). In case of unequal variances (or non-normal distributions), two-tailed Mann-Whitney was used. Data are presented as mean±standard deviation (SD). In the figures, symbols represent statistical significance as indicated: \*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001 and non-significant (NS).

**RESULTS**

**Patient characteristics**

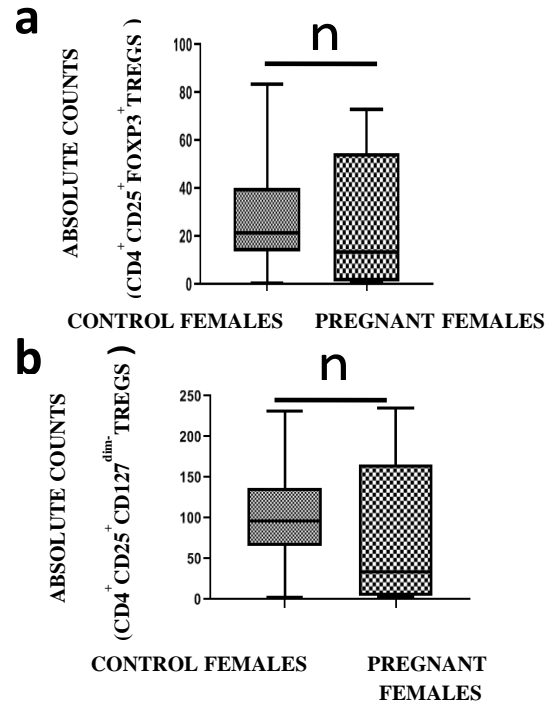
The clinico-physiological characteristics of 100 participants enrolled in this study are listed in Table 1. The participants are categorized according to a particular physiological/pathological state. Out of 100 subjects, 30 were healthy individuals (10 male and 20 female). Different category of subjects include pregnant females (n=10), cord blood (n=10) and patients with various immunopathological conditions such as cancer (newly diagnosed non-Hodgkin's lymphoma, n=10), autoimmune conditions such as (type 1 diabetes mellitus, Hashimoto's disorder, and other autoimmune disorders, n=10 each) and in donor grafts (n=10) following allogeneic hematopoietic stem cell transplant. Other autoimmune disorders include systemic lupus erythematosus (SLE) (n=3), rheumatoid arthritis (n=3), psoriasis (n=1), ulcerative colitis (n=1), pemphigus vulgaris (n=1) and ankylosing spondylitis (n=1).

**Table 1: Details of participants enrolled in the current study.**

Characteristics/status	Number of participants
<b>Sex</b>	
Male (infants – 62 years)	40
Female (infants – 68 years)	60
<b>Healthy controls</b>	
Male	10
Female	20
<b>Pregnant females</b>	10
<b>Cord blood from newborn infants</b>	10
<b>Type 1 diabetes mellitus</b>	10
<b>Hashimoto's disorder</b>	10
<b>Other autoimmune disorders</b>	10
<b>Newly diagnosed non-Hodgkin's lymphoma</b>	10
<b>Donor grafts after allogeneic hematopoietic stem cell transplant</b>	10

**Regulatory T cells in pregnancy**

Accumulating evidence has suggested the role of Tregs as critical players in the maintenance of fetal survival within the maternal uterus.<sup>18</sup> We estimated the levels of absolute Tregs in pregnant (4-14 weeks) and control females. Levels of Tregs (CD4<sup>+</sup> CD25<sup>+</sup> FOXP3, and CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>dim/-</sup>) (Figure 1a and b) in peripheral blood lymphocytes (PBLs) between normal and pregnant females were not significantly different (p>0.05). This could be attributed to small sample size.



**Figure 1: Level of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs and (a) CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs (b) in controls (N=20) and pregnant females (N=10). The values are represented as absolute counts. Values are expressed as mean±SD. Comparisons were performed with control females.**

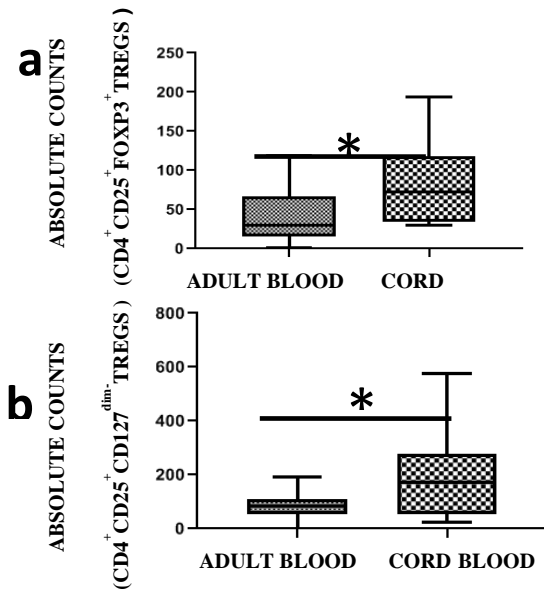
**Regulatory T cells in cord blood**

Regulatory T cells in new-born babies may have a vital role in abrogating excessive immune responses during their initial acclimatization stage.<sup>19</sup> We examined the absolute number of Tregs in 10 new-born babies. We observed significant (p<0.01) augmentation of regulatory T cell numbers (CD4<sup>+</sup> CD25<sup>+</sup> FOXP3 and CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>dim/-</sup>) (Figure 2a and b) in cord blood from newborn infants on comparison with adult PBMCs.

**Regulatory T cells in cancer (non-Hodgkin's lymphoma)**

Plethora of reports has confirmed the existence of increased number of Tregs in tumor microenvironment

which is often correlated with poor prognosis in different cancers.<sup>20</sup> We investigated the difference in the levels of Tregs ( $CD4^+CD25^+FOXP3^+$  and  $CD4^+CD25^+CD127^{dim/-}$ ) (Figure 3a and b) in healthy controls and patients suffering from newly diagnosed non-Hodgkin's lymphoma. However, the difference in levels of both types of Tregs was not significantly different.

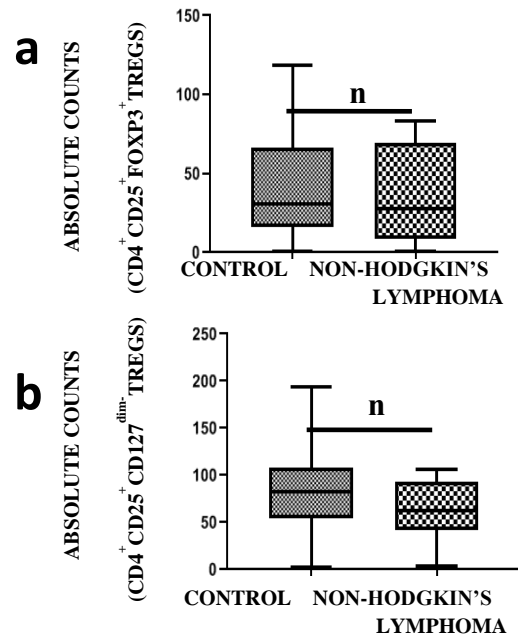


**Figure 2: Level of  $CD4^+CD25^+FOXP3^+$  Tregs and (a)  $CD4^+CD25^+CD127^{dim/-}$  Tregs (b) in adult PBMCs (n=30) and cord blood from new born babies (n=10). The values are represented as absolute counts. Values are expressed as mean±SD. Comparisons were performed with Treg population from adult PBMC.**

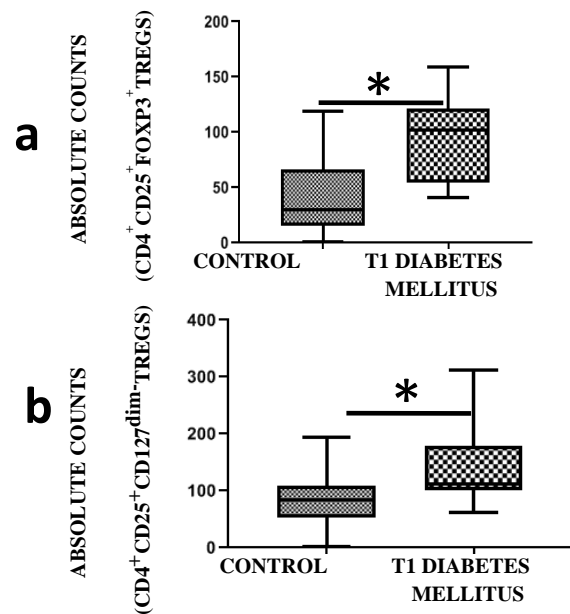
**Regulatory T cells in autoimmune disorders**

There is accumulating evidence that Tregs play a vital role in suppression of several common autoimmune diseases.<sup>8</sup> The distribution of Tregs ( $CD4^+CD25^+FOXP3^+$ ) showed a significant difference ( $p<0.0001$ ) between normal individuals and type 1 diabetes mellitus (T1DM) patients (Figure 4a). Percentage of  $CD4^+CD25^+CD127^{dim/-}$  Tregs (Figure 4b) population was also found to be higher in T1DM patients as compared to normal individuals with a significant statistical difference ( $p<0.01$ ).

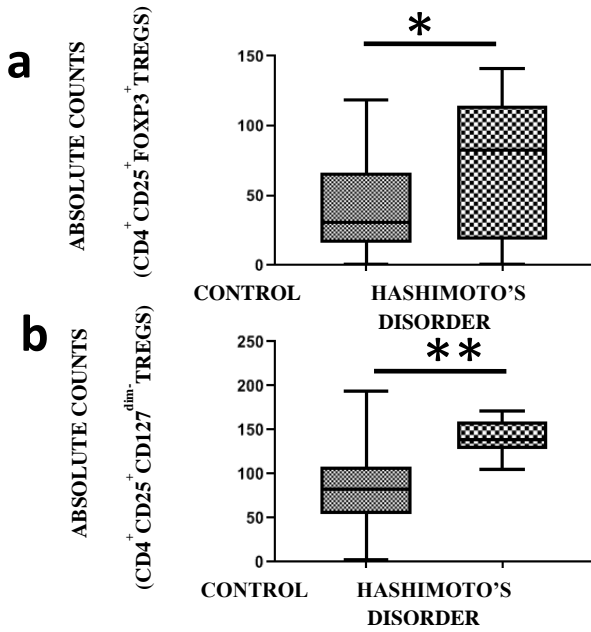
We have also investigated the levels of Tregs in another autoimmune disorder known as Hashimoto's disorder. In our data set, distribution of Tregs ( $CD4^+CD25^+FOXP3^+$  and  $CD4^+CD25^+CD127^{dim/-}$ ) (Figure 5a and b) between healthy controls and patients suffering from Hashimoto's disorder showed significant difference between the groups ( $p<0.05$ ,  $p<0.01$  respectively). However, differences were not that significant in the levels of Tregs ( $CD4^+CD25^+FOXP3^+$  and  $CD4^+CD25^+CD127^{dim/-}$ ) (Figure 6a and b) obtained from healthy controls and patients suffering from other autoimmune disorders.



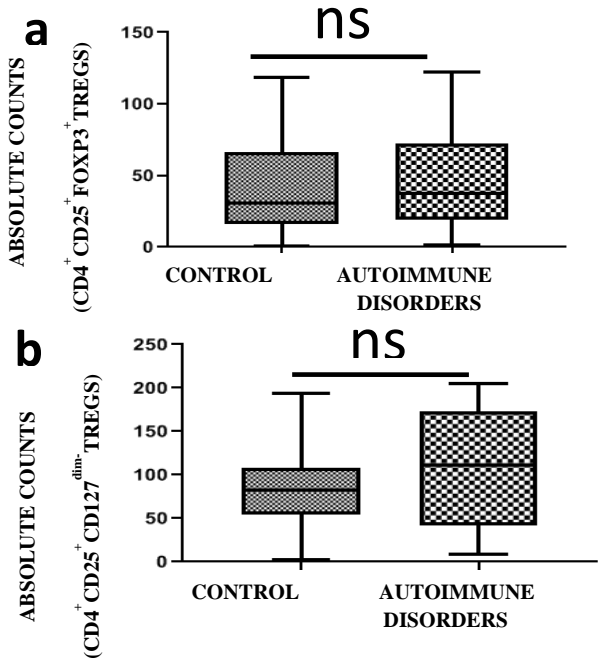
**Figure 3: Level of  $CD4^+CD25^+FOXP3^+$  Tregs and (a)  $CD4^+CD25^+CD127^{dim/-}$  Tregs (b) in controls (n=30) and non-Hodgkin's lymphoma patients (n=10). The values are represented as absolute counts. Values are expressed as mean±SD. Comparisons were performed with Treg population from control individuals.**



**Figure 4: Level of  $CD4^+CD25^+FOXP3^+$  Tregs and (a)  $CD4^+CD25^+CD127^{dim/-}$  Tregs (b) in controls (n=30) and T1DM patients (n=10). The values are represented as absolute counts. Values are expressed as mean±SD. Comparisons were performed with Treg population from control individuals.**



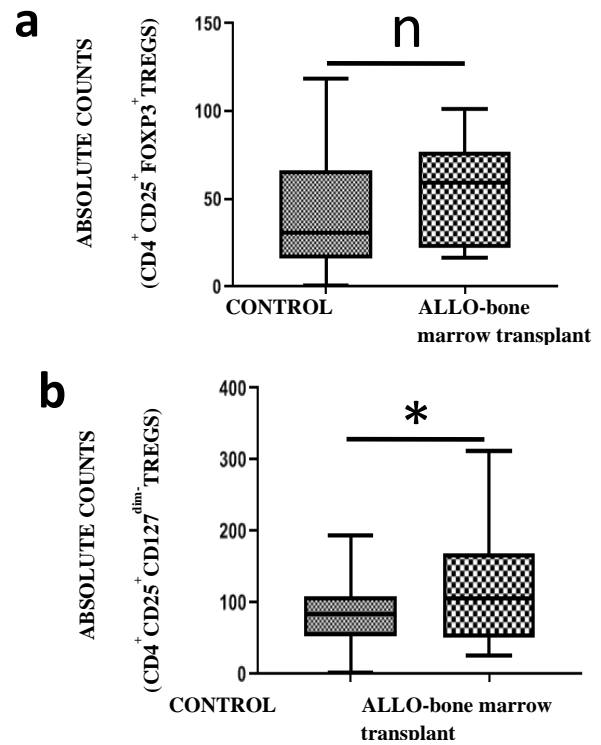
**Figure 5: Level of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs and (a) CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs (b) in controls (n=30) and Hashimoto's disorder patients (n=10). The values are represented as absolute counts. Values are expressed as mean±SD. Comparisons were performed with Treg population from control individuals.**



**Figure 6: Level of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs and (a) CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs (b) in controls (n=30) and autoimmune patients (n=10). The values are represented as absolute counts. Values are expressed as mean±SD. Comparisons were performed with Treg population from control individuals.**

**Regulatory T cells in post allogenic hematopoietic stem cell transplant**

Tregs have been known to play a pivotal role in abrogation of graft-versus-host disease (GvHD).<sup>21</sup> This information intrigued us to examine the levels of Tregs in 10 patients who have received allogenic hematopoietic stem cell transplants and in healthy controls. Tregs were quantified in donor grafts following transplantation. Difference between levels of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>Tregs (Figure 7a) population was not significantly different (p>0.05) between healthy controls and patients. However, levels of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup>Tregs was found to be significantly different (p<0.01) (Figure 7b).



**Figure 7: Level of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs and (a) CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs (b) in control (n=30) and ALLO-bone marrow transplants (n=10). ALLO-bone marrow transplants refers to patients who have received allogeneic bone marrow transplant. The values are represented as absolute counts. Values are expressed as mean±SD. Comparisons were performed with Treg population from control individuals.**

**DISCUSSION**

Growing body of evidence has clearly established the fact that Tregs are known to play pivotal role in the prevention of adaptive immune responses and mechanism of immune tolerance. The immune modulating properties of human regulatory T cells (Tregs) render it effective for novel immunotherapeutic interventions. However, it is crucial to have a better understanding of Tregs biology in various

disease conditions for the development of new therapeutic strategies.

Characterization of Tregs is done by assaying the expression of cell surface proteins CD4 and CD25 and by intracellular expression of FOXP3, a transcription factor. There are two classes of Tregs. Natural (nTregs) originates from thymus and induced (iTregs) from periphery. Previous reports have suggested that FOXP3 is vital for Tregs's suppressive ability making it a critical regulator of Tregs function.<sup>15</sup> The only limitation with FOXP3 is its confined expression in nucleus and hence, it is not useful for identification of nTregs. However, it was also shown that CD25 fails to be called as unique Treg marker owing to its presence on activated T cells also. Recent studies have proposed that diminished expression of CD127 has an inverse correlation with FOXP3 expression and constitutively high expression of CD25 could be used to discriminate Tregs from activated CD4<sup>+</sup> T cells.<sup>14</sup> In our current study, we have analyzed the levels of both types of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup>) in different immuno-physiological conditions.

A big challenge for the maternal immune system is the establishment and maintenance of tolerance against paternal alloantigens expressed in fetal tissues and is responsible for survival of fetus within maternal uterus.<sup>22</sup> Tregs, a subset of suppressor CD4<sup>+</sup> T cells, play a dominant role in the maintenance of tolerance towards the fetus bearing alloantigens.<sup>23</sup> In our present investigation, difference in CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>Tregs and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs levels in peripheral blood lymphocytes (PBLs) between normal and pregnant females were not significantly different. This could be attributed to small sample size. One of the major limitations of our study is the small sample size which is associated with low statistical power.

Tregs regulate the magnitude of immune response via suppression of inflammatory cells. However, Tregs in cord blood are known to prevent immune dysregulation leading to serious disorder such as allergies or autoimmune disorders.<sup>24</sup> Researchers in past have demonstrated the presence of functional Tregs in cord blood mononuclear cells.<sup>25</sup> We examined the number of Tregs in cord blood from 10 newborn infants and compared with adult PBMCs. Levels of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs population in cord blood was significantly higher ( $p < 0.01$ ) as compared to adult PBMCs which is consistent with the findings that Tregs in new-born babies might play a pivotal role in suppressing excessive immune responses because of sudden environmental change.<sup>25</sup>

Tregs contribute to immune evasion by malignancies.<sup>26,27</sup> Tregs have also been shown to suppress anti-tumor responses leading to tumor progression.<sup>28,29</sup> Tregs analysis in 30 normal healthy individuals and 10 non-Hodgkin's lymphoma patients demonstrated non-significant difference between the levels of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> and

CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Tregs. This could be attributed to small sample size which is insufficient when human samples are analyzed. Growing body of evidences has shown augmented increase in Tregs in patients with Non-Hodgkin's lymphoma when compared to healthy controls.<sup>30</sup>

Type 1 diabetes mellitus (T1DM), an autoimmune disorder resulting from self-destruction of insulin producing pancreatic  $\beta$  cell mediated by T cell further leading to hyperglycaemia, and severely impaired glucose homeostasis.<sup>31</sup> Recent reports have suggested that due to imbalance in the levels of Tregs which could one of the reasons for breakdown of peripheral tolerance thus leading to development of T1DM.<sup>32</sup> The distribution of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup>) showed a significant difference ( $p < 0.001$  and  $p < 0.05$ , respectively) between normal individuals and T1DM patients which is contrasting to what researchers have reported about the association of Tregs and T1DM (Figure 4a). However, conclusive statistical analysis should be performed with a larger cohort for further confirmatory validation.

Tregs play a critical role in suppressing autoimmunity which is very well documented by several autoimmune disorders.<sup>33</sup> In our study, we observed significantly increased levels of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup>) in Hashimoto's disorder patients which was quite surprising. However, several contrasting reports are published which have taken into consideration about the increase in the levels of Tregs in tissues which are affected by autoimmunity.<sup>34</sup> Because of complexities of autoimmune human diseases, it is difficult to predict the role of Tregs. The study has to be repeated in large sample size to get any confirmatory observation.

Tregs play an essential role in suppression of graft-versus-host disease (GVHD) and cytomegalovirus (CMV) reactivation thereby abrogating excessive immune responses.<sup>35,36</sup> Acute and chronic GVHD (aGVHD and cGVHD), CMV reactivation and relapse are major barriers for patients undergoing allogeneic hematopoietic stem cell transplant (allo-HSCT).<sup>37</sup> Analysis of Tregs in patients of allo-HSCT, it was observed that number of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>) was elevated in patients than controls. However, the data was not significant. In contrast, significant differences were observed between increased numbers of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup>) in patients on comparison with healthy controls which is consistent with observations that elevated numbers of Tregs is lined to acute graft versus host disease and is essential for recovery of patients after the transplant

## CONCLUSION

Tregs plays an indispensable role for the induction and maintenance of peripheral immune tolerance but they also contribute to different disease conditions such as allergy, autoimmunity, GVHD and cancer. In this study, we have tried to uncover the association of Tregs in different

immune-physiological conditions. This is the first preliminary comparative quantitative analysis of association of Tregs with different diseases. A comprehensive understanding of Tregs biology among various disease and pathological conditions will be instrumental for context specific targeting of Tregs in different modalities. Moreover, it is important to highlight that while working with human diseases or any particular state, it is vital to take large number of samples in a study population in lieu with statistical significance so that the acquired observation can be extrapolated to a particular group.

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*Conflict of interest: None declared*

*Ethical approval: The study was approved by the Institutional Ethics Committee*

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