

**ESTABLISHMENT OF CD4<sup>+</sup> CD25<sup>HI</sup> T CELLS REFERENCE INTERVALS IN HEALTHY ADULT PAKISTANI MALES VERSUS FEMALES**Gul Afshan<sup>1</sup>, Nadeem Afzal<sup>1</sup>, Sadia Qureshi<sup>2</sup>

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**ABSTRACT**

It is generally acknowledged that autoimmune diseases are more prevalent in females all over the world. These diseases are caused by interaction of genetic and environmental factors that result in the failure of immune mechanisms responsible for self-tolerance. Naturally occurring regulatory T cells (T<sub>reg</sub>) prevent autoimmune diseases; nevertheless, gender is one of the important factors that reacts differently to the immune response and therefore constitutes a distinctive potential target for immunotherapy. The aim of the study was to enumerate T<sub>reg</sub> in healthy adult males and females, to find the difference in their frequency between the two genders and to correlate with the established value. T<sub>reg</sub> levels in peripheral blood of 97 young healthy males and females were determined using flowcytometry. Mann Whitney rank sum test was applied to estimate the significance of gender related difference. Significant difference was observed in T<sub>reg</sub> percentages, *p*-value < 0.020 showing that there is lower T<sub>reg</sub> percentage in females than in males (2.89 % ± 1.46 Vs 3.32 % ± 1.39). The modified T<sub>reg</sub> number could render females more prone to autoimmune diseases. The reference ranges of white blood cells have been well laid out for western countries and the same values are being used in Pakistan. The use of these reference values might be misleading since the expected normal values may vary depending upon the race or the geographical region. The estimated values in the present study may contribute to the correct determination of reference values in south Asia and in close proximity regions.

**Key words:** autoimmune diseases, gender, regulatory T cells

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**INTRODUCTION**

Females as a gender group have heightened immune responses not only to foreign antigens but also to self-antigens resulting in a greater preponderance of autoimmune disorders in females than in males [1]. Moreover, the degree of immune response is also more vigorous in females resulting in greater antibody production and increased cell-mediated immunity after immunization. [2] Thus, the sexual dimorphism of immune response acts as a double edge sword making them less susceptible to certain infection but predisposing them to autoimmune diseases. Autoimmune diseases affect approximately 5-8% of the general population and about 78% of these are females [3]. A similar gender bias in the prevalence of autoimmune diseases is also seen in several animal models for autoimmunity.

Autoimmune diseases occur because of failure to eliminate or inactivate autoreactive immunocompetent cells during their ontogeny or due to inability of immune system to control growth and function of self-reactive cells that escape to the periphery [4]. In the periphery, various mechanisms work together to check these absconders and maintain tolerance to self. One of these mechanisms includes  $T_{reg}$  [5, 6]. These regulatory cells represent 0.6-7% of total  $CD4^+$  T lymphocyte population in normal humans [7] and are thought to perform a specialized function of regulating both the innate and adaptive immune function, thus preventing undue damage to self [8].  $T_{reg}$  are antigen specific, but once activated, they become suppressive for the self-reactive immune cells in antigen nonspecific manner [9].

The role of  $T_{reg}$  in preventing autoimmunity has been recorded in different reports on human subjects, which show altered number and/or function of these cells in different autoimmune diseases [10]. Similarly, in many animal models, depletion of  $T_{reg}$  resulted in autoreactivity while reconstitution of these cells prevented development of autoimmune diseases.

As females have a higher incidence of autoimmune diseases and  $T_{reg}$  play a crucial role in preventing autoimmunity, it was reasonable to hypothesize that the females might have lower number of  $T_{reg}$  as compared to males resulting in less effective suppression of auto-reactive lymphocytes and higher incidence of autoimmune diseases in them.

## METHOD

### Study Population

Healthy 19-26 years old 50 males and 47 females of same ethnic group were recruited. The purpose of narrow age range was to exclude the effect of age as a variable. Written, informed consent was obtained. Subjects with any history of acute and chronic infections, known allergic disorders, immunodeficiencies, immunoproliferative and autoimmune diseases, those on long-term anti-inflammatory or immunosuppressive therapy and those with abnormal complete blood count (CBC) were excluded from the study.

### Blood Sample Collection and Processing

3 ml of venous blood was drawn for complete blood count (CBC) and immunophenotyping. Sysmex automated haemalyzer was used for obtaining total leukocyte count (TLC), differential leukocyte counts (DLC) and white blood cell (WBC) percentages. Immunostaining was performed according to the manufacturer's Becton Dickinson (BD) recommendations and cells were analysed with a FACS Calibur 4-color analyzer (BD).

**Sample analysis**

CD4<sup>+</sup>CD25<sup>+</sup> T cells were analyzed using dot-plot graphic method. The percentage of CD4<sup>+</sup> T cells was determined by using gate statistics and their absolute count was computed by multiplying this percentage with the lymphocyte count determined as part of CBC by Haemalyzer.

**Statistical analysis**

Mann Whitney rank sum test was used to determine significant differences between the study groups. P-value < 0.05 was considered statistically significant. (Statistical analysis was done using SPSS version 15)

**RESULTS**

The percentages of CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup> T cells were estimated in both males and females (Table 1). CD4<sup>+</sup> T cells percentage was found to be higher in females than in males (41 ± 7.50 % Vs 39 ± 6.25 %; p-value < 0.001). Conversely, females had lower percentages of CD4<sup>+</sup>CD25<sup>hi</sup> T cells (2.89 ± 1.46 % Vs 3.32 ± 1.39 %; p-value < 0.020). The frequency distribution of these cells in two genders is shown in figures 1 and 2.

No significant gender difference was observed in the percentages and absolute counts of WBC, neutrophils and lymphocytes (Table 2). Similarly no difference was observed in absolute counts of CD4<sup>+</sup> T cells. Comparisons of the values of present study with published values are also summarized in Table 3.

**Table 1: Gender related differences in percentages of different white blood cells\***

| Parameter   | Females<br>n=47              | Males<br>n=50               | p-value† |
|---|------------------------------|-----------------------------|----------|
| Neutrophils   | 56.10 ± 11.22 (40.50 – 68)   | 52.7 ± 10.85 (40.90 – 68.6) | 0.065    |
| Lymphocyte  | 33.2 ± 10.55 (20.90 – 45.40) | 33.1 ± 9.7 (20.90 – 50.30)  | 0.603    |
| CD4 <sup>+</sup> T cells <sup>#</sup>                       | 41 ± 7.50 (34 – 58)          | 39 ± 6.25 (28 – 49)         | 0.001    |
| CD4 <sup>+</sup> CD25 <sup>hi</sup><br>T cells <sup>#</sup> | 2.89 ± 1.46 (1.48 – 6.21)    | 3.32 ± 1.39 (1.77 – 7.13)   | 0.020    |

\* Mann Whitney rank sum test was applied to estimate the significance of gender related difference. Median ± IQR with ranges in parenthesis

†Significant at 5% level of significance

<sup>#</sup>Obtained by flowcytometry

**Table 2: Gender related differences in absolute counts of different white blood cells ( $10^3/\mu\text{L}$ )<sup>\*</sup>**

| Parameter                             | Females<br>n=47            | Males<br>n=50              | p-value <sup>†</sup> |
|---------------------------------------|----------------------------|----------------------------|----------------------|
| WBC                                   | 6.86 ± 1.91 (4.70 – 10.65) | 6.96 ± 2.11 (4.99 – 12.21) | 0.332                |
| Neutrophils                           | 3.74 ± 1.29 (2.06 – 6.81)  | 3.53 ± 1.69 (2.04 – 7.36)  | 0.762                |
| Lymphocytes                           | 2.25 ± 0.67 (1.39 – 3.56)  | 2.26 ± 0.56 (1.57 – 4.61)  | 0.636                |
| CD4 <sup>+</sup> T cells <sup>#</sup> | 954 ± 338 (589 – 1580)     | 892.9 ± 29.2 (547 – 1797)  | 0.187                |

\* Mann Whitney rank sum test was applied to estimate the significance of gender related difference. Median ± IQR with ranges in parenthesis

<sup>†</sup>Significant at 5% level of significance

<sup>#</sup>Calculated from flowcytometry data and absolute lymphocyte count

**Table 3: Comparison of values from the present study with published reference values**

| Parameter                                     | Pakistan     |                | Kenya [36]    |                 | Tanzania [40] |                | Ethiopia [35]           | USA <sup>a</sup>       | India [41]    |                 | China [42]     | Botswana [43]  | Asian [25]     |
|---|--------------|----------------|---------------|-----------------|---------------|----------------|-------------------------|------------------------|---------------|-----------------|----------------|----------------|----------------|
|   | Male<br>n=50 | Female<br>n=47 | Male<br>n=484 | Female<br>n=445 | Male<br>n=42  | Female<br>n=60 | Both<br>gender<br>n=485 | Both<br>gender<br>n=NA | Male<br>n=120 | Female<br>n=132 | Both<br>gender | Both<br>gender | Both<br>gender |
| CD3 <sup>+</sup> T cells/ul                   | NA           | NA             | 1293          | 1660            | 683-2106 †    |                | 854-2556 †              | 723-2737 †             | 1554          | 1640            | NM             | NM             | NM             |
| CD3 <sup>+</sup> T cells %                    | NA           | NA             | 72            | 74              | 52-80 †       |                | NA                      | 56-86 †                | 71.6          | 74.5            | NM             | NM             | NM             |
| CD4 <sup>+</sup> T cells/ul                   | 892.9        | 954            | 744           | 982             | 596.5         | 764.5          | 366-1235 †              | 404-1612 †             | 794           | 906             | 727 *          | 726 *          | 838 *          |
| CD4 <sup>+</sup> T cells %                    | 39           | 41             | 41            | 44              |               |                | NA                      | 33-58 †                | 36.89         | 41.38           | NM             | NM             | 35.6 *         |
| CD8 <sup>+</sup> T cells/ul                   | NA           | NA             | 454           | 549             | 417           | 513            | 311-1618 †              | 220-1129 †             | 645           | 626             | 539 *          | 488 *          | 642 *          |
| CD8 <sup>+</sup> T cells %                    | NA           | NA             | 26            | 25              |               |                | NA                      | 13-39 †                | 29.65         | 28.51           | NM             | NM             | 27 *           |
| CD4:CD8 ratio                                 | NA           | NA             | 1.6           | 1.7             | 1.6           | 1.5            | 0.4-2.4 †               | NA                     | 1.3           | 1.58            | 1.49 *         | 1.57 *         | 1.43 *         |
| Lymphocytes /ul                               | 3310         | 3320           | 1860          | 2160            | NM            | NM             | NM                      | NM                     | 2198 *        |                 | NM             | NM             | 2375 *         |
| Lymphocytes %                                 | 22.6         | 22.5           | 45            | 45              | NM            | NM             | NM                      | NM                     | 31.35         | 31.16           | NM             | NM             | NM             |
| CD4 <sup>+</sup> CD25 <sup>hi</sup> T cells % | 3.32         | 2.89           | NA            | NA              | NA            | NA             | NA                      | NA                     | NA            | NA              | NA             | NA             | NA             |

\* Total mean value of study population irrespective of gender

<sup>†</sup> Value in lower and higher ranges irrespective of gender

<sup>a</sup>: Reference ranges provided by Becton-Dickinson with the MultiTEST IMK Kit Reagent package (12/2000; 23-3602-02).

NA - Not available

NM - Not mentioned

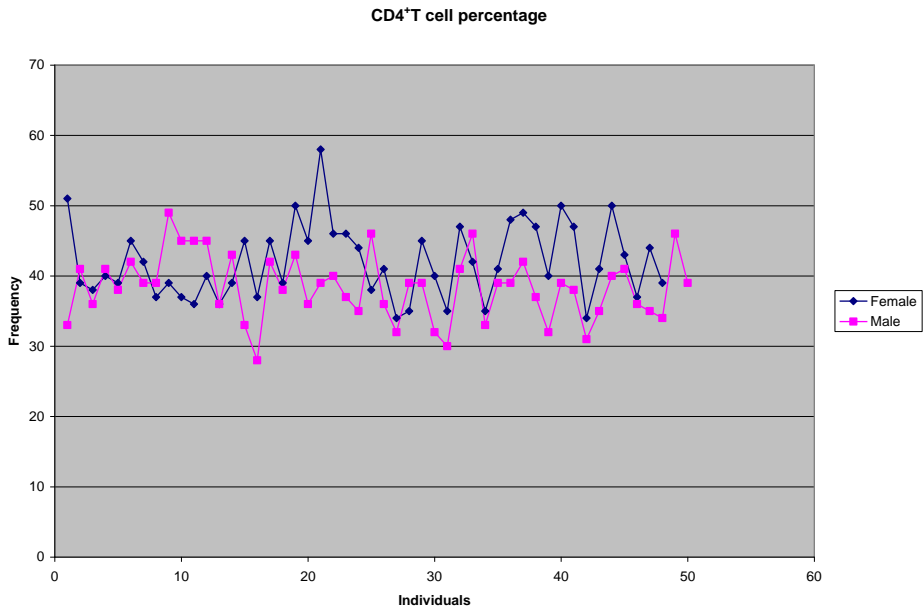


Figure 1: Frequency distribution of CD4+ T cell percentages among males and females

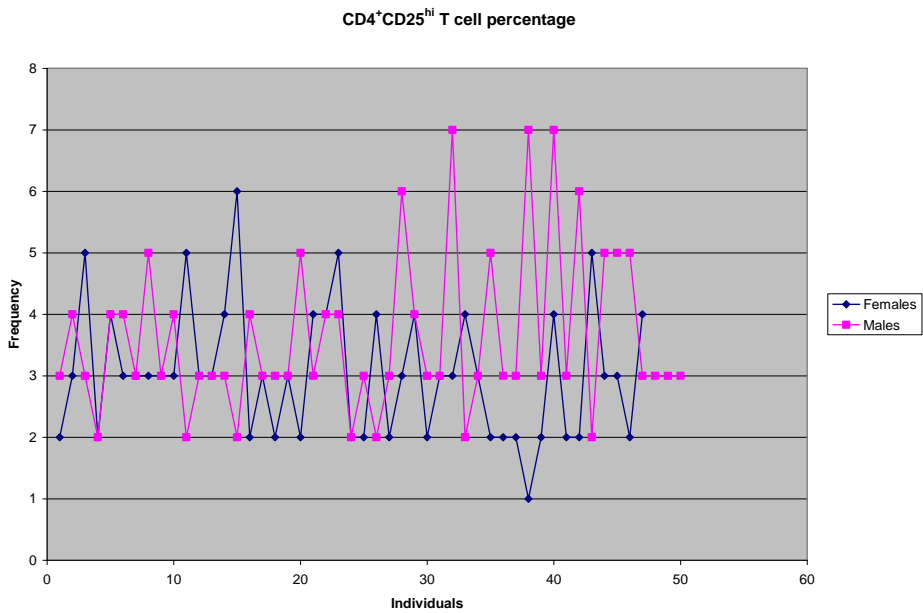


Figure 2: Frequency distribution of CD4+CD25hi T cells as percentage of total CD4+ T cells among males and females

## DISCUSSION

Results of this study reveal that healthy females have lower percentage of  $T_{reg}$  in their peripheral blood. This confirms the assumption that  $T_{reg}$  may partly contribute to gender differences in autoimmune diseases among males and females. One of the studies reported that  $T_{reg}$  contribute to gender differences in susceptibility of experimental autoimmune encephalomyelitis in mice [11]. It is further supported by a recently published study which demonstrated lower number of  $T_{reg}$  in healthy females compared to healthy males [12]. The study also demonstrated 3–4-fold higher Foxp3 mRNA expression in  $T_{reg}$  of healthy males as compared to healthy females [12]. In contrast to the results of the present study, another study in humans showed comparable number of  $T_{reg}$  among healthy males and females [13]. However, in that study sample size was so small that very little conclusion could be drawn. Still the difference in the results could be due to the difference in methodology. Moreover, the difference of environment and living conditions, degree of exposure to infectious agents and genetic and racial factors could be the cause of this disparity in results. The other studies that demonstrate comparable  $T_{reg}$  number in patients with autoimmune polyglandular syndrome type II, type I diabetes, psoriasis and myasthenia gravis have shown  $T_{reg}$  functional defects [14].

Reference ranges are crucial for interpretation of hematological data and for deriving meaningful information in clinical laboratory. The reference ranges of blood cells in peripheral blood of healthy individuals have been well laid out for western countries and the same values are being used in Southeast Asia as well. Lately, there have been attempts to determine these reference values in Asia and Africa [15-18]. From CBC, the ranges of absolute counts and percentages of WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils,  $CD4^+$  and  $CD4^+CD25^{hi}$  T cells with mean and median in the study population were estimated. These values may contribute to the determination of reference values of haematological parameters in Pakistani population area.

WBC and neutrophil absolute counts of this study were found to be comparable to those for Caucasian population but these are higher than reported for Africans (Table 3). This result is consistent with the previous study among four ethnic female groups in the United Kingdom [19]. The absolute counts of peripheral lymphocytes observed in this study are higher to those found in Indian, Saudi and Kenya studies [15, 20-21]. The percentages of peripheral lymphocytes cells observed in this study are less to those of Indians but higher in Kenya population. With regard to gender related differences, male to female comparison shows no difference in the absolute counts and a percentage of peripheral lymphocytes that is in agreement with previous studies [19-21].

The absolute counts of  $CD4^+$  T cells observed in this study are at variance with those obtained in Indian, Chinese and African populations. These studies showed lower absolute counts of  $CD4^+$  T cell than those obtained in this study [15-16, 20-21, 22-23]. However, in one Ugandan study, these values were reported to be higher than those

seen in this study [24]. Similarly, the percentages of CD4<sup>+</sup> T cells observed in this study are different from those of USA but similar to Kenya, Indians, and Caucasians [15-16, 21, 25]. Thus, numbers recorded here are higher than among the USA, Indians and Chinese, whereas they are similar to numbers reported for Kenya (Table 3).

Our results also demonstrate that there is significant difference in CD4<sup>+</sup> T cell percentages among females and males, their mean and median being higher in females. This is consistent with previous studies, which have shown higher CD4<sup>+</sup> T cells percentages in females [14-15, 18-20, 23]. Though the absolute number of CD4<sup>+</sup> T cells was noted to be higher in females, this difference did not reach statistical significance. This is somewhat in contrast to previous reports that showed significantly higher counts among females as compared to males [15-16, 18-20, 23-25]. The limitation of the study was the differences in T<sub>reg</sub> numbers among different races were not depicted.

### CONCLUSION

A significant difference in the frequency of T<sub>reg</sub> among males and females were identified, which could be one of the reasons for increased tendency of females to autoimmune diseases. It is also identified that there is heterogeneity in the data in different ethnic populations. Therefore, it is important to have baseline values on hematological indices of normal healthy individuals from different ethnic groups in order to define pathologic conditions, facilitate the correct interpretation of results, and thus making right decision in the treatment of the patients.

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