Enumeration of $CD4^+CD25^+$ T regulatory cells in Type-II diabetes retinopathy

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Abstract: Diabetes mellitus (DM) is a health concern because it leads to complications such as retinopathy. Pakistan has 6.9 million DM affected people that will be doubled by 2025. A study was designed to enumerate $CD4^+CD25^+$Treg cells in Pakistani type 2 diabetes mellitus (T2DM) patients. It was a cross-sectional case-control study that included 212 subjects. The subjects having diabetic retinopathy were labeled as Group-I (30 healthy volunteers without diabetes), Group-II (30 T2DM patients without retinopathy) and Group-III (152 T2DM patients with retinopathy). The percentage of $CD4^+CD25^+$ Treg cells was determined by Flowcytometry. Comparison of $CD4^+CD25^+$T cells among different groups was not significant and higher percentage of Treg cells was observed in Group-II (3.07%) compared to Group-III (2.88%). Age, gender and duration of diabetes may contribute while percentage of $CD4^+CD25^+$T cells and Treg cells were not associated with the development of DR in T2DM.

Keywords: T cells, treg cells, autoimmunity, diabetes mellitus.

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease which leads to many complications such as retinopathy, nephropathy, and cardiovascular disease and it is present all over the world. Hyperglycemia, diet, sedentary life-style, age, obesity and genetic profile are associated with diabetic complications. DM is affecting 240 million people worldwide and it may increase up to 380 million by 2025, and more than 80% of diabetics are in low and middle income countries Danaei *et al* (2009). In Pakistan; 6.9 million people were affected with diabetes and it might affect more than 11.5 million people by the year 2025 (Qidwai *et al* 2010). Diagnosis of DM should include determination of fasting blood glucose level and after two hours of 75g glucose load (World Health Organization Expert Committee 1999).

Type-2 DM (T2DM) is relatively more common and is a major cause of morbidity and mortality all over the world. Currently, T2DM is considered as an epidemic of young people and children of 8 years or younger are being affected with this disease. Rise of T2DM is associated with increased prevalence of obesity which is linked to change in dietary and lifestyle patterns. In 20 years' T2DM may account for the 60% of disease load in non-communicable disease group in the developing world. Genetic and environmental factors are blamed for T2DM and up to 25% of first degree relatives of T2DM patients may develop this disease (Dedoussis *et al* 2006).

Asians are less obese and overweight compared to Western people but Asians have similar or even higher prevalence of DM. Increased childhood obesity, abdominal obesity and low muscle mass with increased insulin resistance in Asians put young individuals at high risk to develop T2DM (Chan *et al* 2009).

Prolonged hyperglycemia leads to alterations in retinal and renal blood flow that causes changes in microcirculation that leads to diabetic retinopathy and nephropathy (Tong *et al* 2008). Diabetic retinopathy (DR) is a horrifying complication of diabetes and the people at risk to develop DR would be doubled in 30 years. Clinically, DR is diagnosed by micro aneurysms, hemorrhages and cotton wool spots. New vessels are fragile and lack normal barrier function; therefore they allow extravascular leakage of different components of blood (Funatsu *et al* 2005).

Individuals with raised inflammatory markers are likely to develop T2DM which suggests that inflammation persist much before the diagnosis of T2DM is made (King *et al* 2008). In DR patient, there are anti-pericyte and anti-endothelial cell antibodies. There is increased intra vitreous concentration of IL-6 and IL-8 while raised serum level of IL-8, TNF-alpha and IL-2 receptor is observed. Diabetic preretinal membranes have deposition of immunologlobulins, monocytes, components of activated complement system, suppresser and cytotoxic T lymphocytes. There is aberrant expression of human leukocyte antigen-DR and DQ on retinal vascular endothelial cells. These findings suggest autoimmune process during DR (Kastelan *et al* 2007).

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T regulatory (Treg) cell is a subset of $CD4^+$T cells that maintain peripheral tolerance and down regulate antigen specific immune responses by secreting TGF-β, IL-10 and IL-4 to stop autoimmunity (Madakamutil *et al* 2003). In periphery Tregs express CD25 and forkhead winged-helix family transcription factor (Foxp3) which is a master control gene. In humans $CD4^+CD25^+$T cells repertoire is quite diverse and reduced level of TCR on Treg cells guide its activation. There is insufficient suppression of inflammatory process or transformed Treg cells for initiation and later for continuation of autoimmune disease (Dejaco *et al* 2005).

There is no specific surface marker for enumeration of Treg cells but expression of IL-2 receptor alpha chain (CD25) is a reliable marker. Other markers include CTLA-4, CD62L (L-selectin), CD134 (OX40), glucocorticoid induced tumor necrosis factor receptor, membrane bound TGF-β, programmed cell death-ligand 1, and α4β7/α4β1 integrin. It was suggested that better means for identification, prevention and treatment of DR should be developed before the onset of vision loss (Hoffmann *et al* 2007, Dejaco *et al* 2005).

Since the exact cause of DR is not known and the involvement of autoimmune process is also questionable, therefore a study was designed to enumerate Tregs cells in type-II DR.

MATERIAL AND METHODS

It was an analytical cross-sectional study, performed in the Department of Immunology, University of Health Sciences Lahore. It included 212 subjects i.e. 30 healthy controls (Group-I), 30 diabetic patients without retinopathy (Group-II) and 152 diabetic patients with retinopathy (Group-III). Patients had diabetes between 5yrs–25yrs. Percentage of HbA1c and duration of diabetes was noted at the time of blood sample collection. Eye examination/status of DR was made by consultant ophthalmologist. Patients were from either sex and between 20-75 years of age. Subjects with a history of infection in the last two weeks and chronic infection like TB, autoimmune disorders, etc. were excluded. To avoid confusing effects of impaired kidney functions, patients of nephropathy were excluded. $CD4^+CD25^+$Tregs were enumerated by FACS Calibur 4-color analyzer (Becton Dickinson: BD) using monoclonal antibodies from BD. Venous blood (2-3m) in EDTA tube was collected.

The methodology was opted from Afshan *et al* (2012) except washing step. Lyse-wash method on whole blood was used and for each sample 100ul was added into 2 BD Falcon tubes. 20ul each of Fluorescein isothiocyanate (FITC) tagged with CD4, phycoerythrin (PE) with CD25 and peridinin-chlorophyll-protein (PerCP) with CD45 cells were added to one tube and 20ul of isotype control to other. The instrument was calibrated and fluorescent signal compensation was performed by using Cell Quest Pro software (BD) and Calibrite beads (BD).

Data acquisition and sample analysis

It was similar to Afshan *et al* (2012) and gating strategy to identify $CD4^{dim}CD25^{bright}$ T cells was adopted by Hoffman *et al* (2007). Using these strategies, quadrants for CD4 and CD25 cells were defined and their percentages were determined and then percentage of Treg cells was calculated.

STATISTICAL ANALYSIS

Data was analysed using SPSS 17.0. One way ANOVA and Post Hoc Tukey test to observe group means differs, Pearson correlation test to observe correlation between quantitative variables and Pearson Chi-Square test to observe association between qualitative variables. A *p*-value of ≤ 0.05 was considered statistically significant.

Ethical consideration

The study was approved by the Ethical Committee and Advanced Studies and Research Board of University of Health Science Lahore. Informed consent was obtained from each participant after explaining details of research.

RESULTS

The demographic data of the studied population is shown in table 1. The comparison of different variables among the three groups is shown in table 2. The comparison of gender distribution among three groups was statistically significant (*p*-value=0.0029). More females were found in Group-II (83%) and in Group-III (66%) compared to Group-I (30%) (*p*-value <0.0001 in each). The comparison of age among three groups was statistically significant (*p*-value<0.0001). Higher age was found in Group-II (49yrs) and in Group-III (50yrs) compared to Group-I (34yrs) (*p*-value<0.0001 in each).

The comparison of $CD4^+CD25^+$T cells among different groups was not significant. From the $CD4^+CD25^+$T cells, percentage of T-reg cells was calculated. On comparison of Treg cells between Group-II and Group-III, higher percentage of Treg cells was found in Group-II (3.07%) compared to Group-III (2.88%) (*p*-value=0.0150).

Logistic regression model for Group-II and Group-III and for Group-I and Group-III was applied to determine association among various variables. Between Group-II and Group-III, there was significant difference in the percentages of Treg cells (*p*-value=0.039) and the number of $CD4^+CD25^+$ cells (*p*-value=0.05). Age and percentages of $CD4^+$ $CD25^+$ T cells were found significant predictors between Group-I and Group-II (*p*-value<0.0001 and 0.004 respectively).

Table 1: Demographic data of the subjects

Variables/Parameters	Group-I (30)	Group-II (30)	Group-III (152)
Male n (%)	21(70)	05 (16.66)	51 (33.55)
Female n (%)	09 (30)	25 (83.33)	101 (66.44)
Age (yrs) range mean ± SD	24-64 34.66±8.78	27-75 49.46±9.94	20-70 50.88±8.90
HbA1c (percentage) mean ± SD	NA*	5.9-12.6 8.54±2.06	5.2-15.4 8.83±2.35
Duration of diabetes (range)			
5 – 10 years n (%)	NA*	25 (11.79)	84 (39.62)
More than 10 years (%) mean ± SD	NA*	05 (2.35) 7.76±4.14	68 (32.07) 10.51±5.24

*NA= not applicable

Table 2: Comparisons of different variables in different groups

Variable	Group-I (n=30)	Group-II (n=30)	Group-III (n=152)	*p*-value
Gender Male (n, %) Female (n, %)	 21 (70) 9 (30)	 5 (16.6) 25 (83.33)	 51 (33.55) 101 (66.44)	0.0029*[1] <0.0001*[2] <0.0001*[3] 0.0868[4]
Age (yrs) min max Mean ± SD	24.0 64.0 34.66±8.78	27.0 75.0 49.46±9.94	20.0 75.0 50.88±8.90	<0.0001*[1] <0.0001*[2] <0.001*[3] 0.4365[4]
Duration (yrs) min max Mean ± SD	NA#	5.0 20.0 7.76 ± 4.14	2.0 26.0 10.51±5.24	0.0073*[4]
HbA1C (bands) min max Mean ± SD	NA#	5.90 12.60 8.54 ± 2.06	5.20 15.40 8.83±2.35	0.6044[4]
CD4CD25 (%) min max Mean ± SD	 7.08 25.64 14.53±4.84	 1.60 27.55 14.68 ± 6.21	 2.14 31.54 16.47±6.56	0.0675[1] 0.8374[2] 0.1272[3] 0.1705[4]
T-regs (%) min max Mean ± SD	2.28 - 4.40 2.91±0.04	2.36 - 4.02 3.07±0.43	2.23 - 4.72 2.88±0.38	0.2434[1] 0.5383[2] 0.6643[3] 0.0150*[4]

*Statistically significant
NA=not applicable, [1]Comparison among three groups, [2] Comparison between group-I and group-II, [3] Comparison between group-I and group-III, [4] Comparison between group-II and group-III

Table 3: Logistic Regression Model for Group-II and Group-III

Variable	Degree of Freedom (DF)	Estimate	Standard Error	Chi-Square	*p*-value
Age	1	0.0004	0.0008	0.21	0.644
Duration	1	0.0019	0.0015	1.62	0.203
HbA1C	1	0.0006	0.0034	0.04	0.847
CD4CD25	1	0.0022	0.0011	3.59	0.058
Tregs	1	-0.0419	0.0204	4.24	0.039*
Logistic Regression Model for Group-I and Group-III					
Age	1	0.0174	0.0027	42.93	<0.0001*
CD4CD25	1	0.0099	0.0035	8.12	0.0044*
T-regs	1	0.0383	0.0603	0.40	0.5249

* statistically significant ($p \leq 0.05$)

DISCUSSION

In the current study, on comparison of gender distribution, there was significant difference among three groups, between Group-I and Group-II, and between Group-I and Group-III (*p*-value=0.0029, <0.0001, <0.0001) respectively but there was no significant difference between Group-II and Group-III. The possible explanation for non-significant value could be that in both the groups' percentage of female subjects was more compared to male subjects. The current study included more females compared to males' i.e. 63.67% females and 36.32% males which is in agreement with Akram *et al* (2011), Chhutto *et al* (2009), Ahmadani *et al* (2008), Jamal-u-Din *et al* (2006), and Tam *et al* (2008).

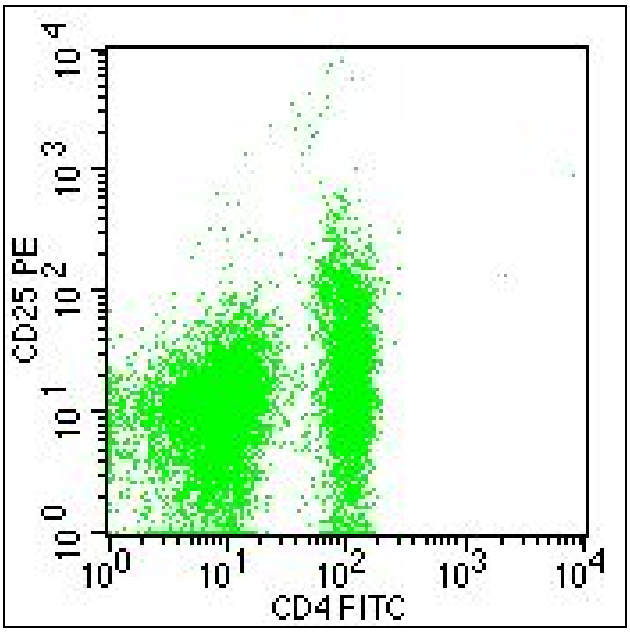

Fig. 1: CD4-CD25 two parameter dot-plot showing that CD4$^+$CD25$^+$ T cell population is not discernable from CD4$^+$CD25$^-$ T cells

Findings of Khan *et al* (2007), and Qidwai *et al* (2010) are different from the current study as they documented higher prevalence of diabetes in males (1.01%) compared to females (0.76%) however impaired glucose tolerance is higher among females compared to males (Shera *et al* 2007). In the literature, majority of studies have documented more prevalence of diabetes in females compared to males and the current study is in agreement with them. Studies in Pakistan showed impaired glucose tolerance as 6.3%-6.9% in men and 10.9%-14.2% in women (Ahmadani *et al* 2008). Similarly, National Diabetic Survey of Pakistan suggested higher prevalence of diabetes in males compared to females while there is higher prevalence of impaired glucose tolerance in females compared to males (Shera *et al* 2007). Nevertheless, Khan *et al* (2007) documented higher prevalence of diabetes in males (1.01%) as compared to females (0.76%) in certain districts of Pakistan.

In the current study, highest mean ±SD of age was detected in Group-III (50±8.90yrs), followed by Group-II (49±9.94yrs) and Group-I (34±8.78yrs). On comparison among three groups, there was significant difference in age (*p*-value<0.0001). Higher mean±SD of age was observed in Group-II (49±9.94yrs) compared to Group-I (34±8.78yrs) (*p*-value<0.0001) and higher mean±SD of age was observed in Group-III (50±8.90yrs) compared to Group-I (34±8.78yrs) (*p*-value<0.0001). There was no significant difference between mean±SD of age of Group-II and Group-III. Possible explanation for no difference in mean±SD of age range could be that both groups had diabetic patients but diabetic retinopathy could be due to other factors e.g. genetics and environmental factors that need to be investigated in future studies (Silverman *et al* 1995).

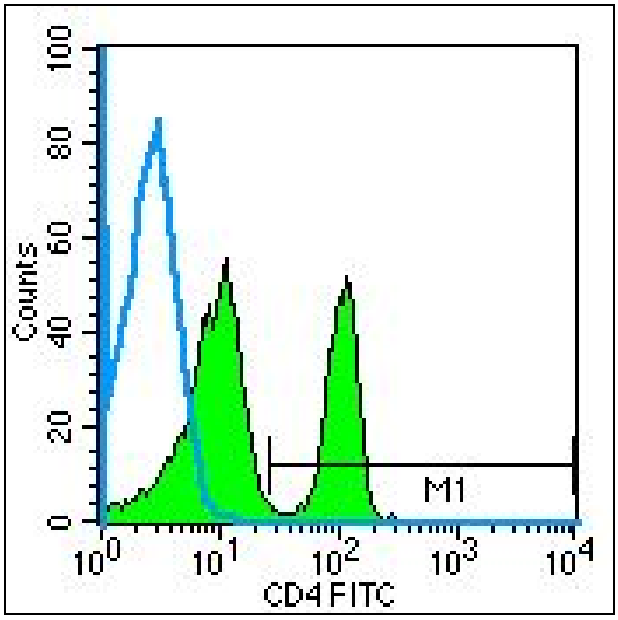

Fig. 2: Determination of criteria for CD4 negative and CD4 positive cells.

Regarding age distribution, current study is in agreement with Chhutto *et al* (2009), Ahmadani *et al* (2008), Shaw *et al* (2010), and Tam *et al* (2008). However study of Akram *et al* (2011) is not in agreement with current study because they set lower age limit as 40 years for diabetic patients while they did not set maximum age limit. The study of Zhang *et al* (2010) is also different because they included patients between 58.9-62.9 years.

In the current study percentage of mean ±SD of HbA1c was 8.54%± 2.06 and 8.83%±2.35 in group-II and group-III respectively. On comparison there was no significant difference. Ahmadani *et al* (2008) and Tam *et al* (2008)

are in agreement with current study, as they also could not observe significant difference in percentage of HbA1c. The studies of Zhang *et al* (2010) and Yang *et al* (2010) are not in agreement with current study as they could determine significant difference in percentage of HbA1c among diabetic patients.

In the current study, almost similar levels of HbA1c in both groups reflect poor diabetes control. The reason could be that these patients had disease for more than 5-years, and they were recruited from public hospital/non-governmental organizations. Majority of patients were from low socio-economic background and their education level was not high. Education and socioeconomic status has been associated with increased prevalence of diabetes (Seeman *et al* 2008).

Regarding duration of diabetes, current study showed significant difference in two groups and it is in agreement with Ahmadani *et al* (2008) and Zhang *et al* (2010). The current study is not in agreement with Jamal-u-Din *et al* (2006) and Tam *et al* (2008), because they included newly diagnosed–up to 15 years and 8.8±6.1 years respectively. The possible explanation for significance of duration of diabetes could be that along with other factors, length of disease could play a role in development of diabetic retinopathy.

In the current study, on comparison of $CD4^+CD25^+$ cells there was no significant difference. Similar results were found by Sun *et al* (2010), Liu *et al* (2008), Brusko *et al* (2005), and Baecher-Allan *et al* (2001). Although these studies detected $CD4^+CD25^+$ T cells in similar range as in the current study but these studies should not be compared with current work because none of them was performed in type-II diabetes patients. Radstake *et al* (2009), Abdulahad *et al* (2007), Yeh *et al* (2006), and Smyth *et al* (2007) are different from the current study. In the current study, reason for not detecting significant difference in $CD4^+CD25^+$ cells could be that these cells are not involved directly in development of DR in type-II diabetes.

In the current study, higher percentage of Treg cells was determined in Group-II (3.07%) compared to Group-III (2.88%) (*p*-value=0.0150) but there was no significant difference of Treg cells among the three groups, between Group-I and Group-II, and between Group-I and Group-III. Therefore, in the current study direct involvement of Treg cells in development of DR was not documented.

Studies on Treg cells in agreement with current work include Radstake *et al* (2009), Liu *et al* (2008), Chen *et al* (2007), Brusko *et al* (2005), Baecher-Allan *et al* (2001), Guyot-Revol *et al* (2006). There are studies that are not in agreement with current work such as Sun *et al* (2010), Hoffmann *et al* (2007), and Abdulahad *et al* (2007). The results of above mentioned studies cannot be compared with the current study because none of them were performed on T2DM. Although, there are a few studies of Treg cells in type-I diabetes (D'Alise *et al* 2008, Clough *et al* 2008, Tong *et al* 2008) but studies of Treg cells in diabetes type-II is very limited. Possible explanation for not finding significant difference in percentage of Treg cells could be that Treg cells are not associated with DR in T2DM.

CONCLUSION

Age, gender of subjects and duration of diabetes may contribute while level of $CD4^+CD25^+$T cells and percentage of Treg cells are not associated with the development of DR in T2DM.

SUGGESTIONS

Highest level of $CD4^+CD25^+$ T cells while highest percentage of Treg cells was observed in diabetes with retinopathy and in diabetes without retinopathy respectively but these findings could be due to disease manifestations. Functional assays of $CD4^+CD25^+$ T cells should be performed in DR of T2DM and more reliable markers for enumeration of Treg cells such as Foxp3 or CD127 should be used.

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