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RESEARCH ARTICLE

Frequency of $CD4^+CD25^{hi}FOXP3^+$ Regulatory T Cells in Type 1 Diabetes Mellitus

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Abstract

Background: Regulatory T cells (Tregs) characterized by CD4, CD25, and transcription factor forkhead box P3 (FOXP3) play a crucial role in maintaining peripheral tolerance. Type 1 diabetes mellitus (DM1) is characterized by destruction of pancreatic islets by auto-reactive T cells. Recently, the role of Tregs in the pathogenesis of DM1 has been interrogated.

Objectives: The current study was conducted to determine the percentage of $CD4^+CD25^{hi}FOXP3^+$ Tregs in patients with DM1.

Patients and methods: This case-control study included 32 patients suffering from DM1 selected from the Outpatient Clinic of Diabetes Mellitus of the Specialized Internal Medicine Hospital, Mansoura University, Mansoura, Egypt. Further 24 age- and gender-matched healthy subjects were enrolled as a control group. Flow cytometric analysis was performed for evaluation of the percentage of $CD4^+CD25^{hi}FOXP3^+$ Tregs in the peripheral blood samples of patients with DM1, as well as control subjects.

Results: Patients with recent onset DM1 had reduced percentage of $CD4^+CD25^{hi}FOXP3^+$ Tregs compared to patients with established disease [$p = 0.02$], and control subjects [$p = 0.0001$]. Nevertheless, no statistically significant difference was detected between patients with established disease and control subjects [$p > 0.05$].

Conclusion: The percentage of $CD4^+CD25^{hi}FOXP3^+$ Tregs is reduced in patients with DM1 compared to control subjects. However, these findings need to be evaluated in a larger cohort of patients to validate results.

Introduction:-

Regulatory T cells (Tregs) play a paramount role in the development and maintenance of immunological tolerance **[Itoh et al., 1999]**. The history of Tregs in the immunology literature dates back to the 1970s, when T lymphocytes that were capable of suppressing immune responses were first described, and coined "suppressor T cells" **[Gershon et al., 1972]**. Nonetheless, the advent of modern-day Treg research can be dated to 1995, when Sakaguchi et al. first described the suppressive function of $CD4^+CD25^+$ Tregs in mice, and showed that depletion of these cells resulted in multi-organ autoimmune disorders on adoptive transfer **[Sakaguchi et al., 1995]**.

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In healthy individuals, $CD4^+$ Tregs account for 5-10% of the total $CD4^+$ T cell population **[Baecher-Allan et al., 2001]**. Strikingly, 2-3% of these cells are characterized by high expression levels of CD25 ($CD25^{high/hi}$), and possess a potent suppressive activity **[Fehervari and Sakaguchi, 2004]**.
Work in the field of Tregs' immunology was greatly upgraded in 2003 by the discovery of Treg-specific gene, *forkhead-box P3 (FoxP3)* which encodes for FOXP3 protein **[Ochs et al., 2007]**. This protein has been characterized as the master regulator of the development and function of Tregs. It is a member of the forkhead/winged-helix family of transcription factors, based on its winged-helix/forkhead DNA-binding domain **[Coffer and Burgering, 2004]**.

Type 1 diabetes mellitus (DM1) is principally a T cell-mediated autoimmune disease characterized by the destruction of pancreatic beta (β) cells leading to insulin deficiency. The role of genetic predisposition, immune dysfunction, as well as environmental triggers in disease development has been questioned **[Atkinson and Eisenbarth, 2001]**. While the exact pathogenic mechanisms leading to DM1 still remain largely obscure, it has been assumed that the presence of a defective Tregs' population in patients with DM1 contributes to diabetes development **[Brusko et al., 2005]**.

A plethora of studies have investigated the potential role of $CD4^+CD25^{hi}FOXP3^+$ Tregs in the aetiopathogenesis of DM1, however, these studies yielded discordant results **[Paul et al., 2015]**. So, in the current work, we aim at addressing the role of Tregs in DM1 by analyzing the percentage of $CD4^+CD25^{hi}FOXP3^+$ Tregs in patients with DM1 as compared to healthy control subjects.

Subjects and methods:-

This is a case-control study, conducted over a period of 12 months starting from March 2014 to February 2015.

Subjects:-

- The recruited subjects included 32 patients with DM1 (19 females, and 13 males), with age ranging between 18 and 25 years (mean age; 22.2 ± 3.5 years) attending the Outpatient Clinic of Diabetes Mellitus of the Specialized Internal Medicine Hospital, Mansoura University, Mansoura, Egypt. DM1 was diagnosed according to American Diabetes Association criteria **[*American Diabetes Association, 2003*]**.
- Further 24 age (mean age; 19 ± 4.1 years) and gender (F/M; 15/9) matched subjects were enrolled as a healthy control group. They had no history of autoimmune diseases. Besides, history of DM1 in their families was ruled out as confirmed by medical records, and laboratory investigations.
- Patients were further subdivided into 2 groups; group 1 (n= 17) comprising patients with recent onset DM1 (< 12 weeks from diagnosis), and group 2 (n= 15) encompassing patients with established DM1 (> 12 weeks duration).
- All patients were under treatment with humanized insulin at doses of 0.84 ± 0.2 units/kg/day.
- In all patients, the C-peptide levels were < 0.5 ng/ml.
- In addition to insulin, 8 patients were on antihypertensive drugs and 6 on lipid-lowering medications.
-The medical examination included medical history and physical examination. Body mass index (BMI) was calculated as kg/m^2. Mean blood pressure (MBP) was calculated as diastolic blood pressure + 33% of pulse pressure.
- Laboratory analyses included: complete blood count (CBC), fasting blood glucose (FBG), glycosylated haemoglobin (HbA1c), lipid profile, and serum creatinine.
- Patients with concomitant autoimmune disorders were excluded from the study.

Laboratory workup:-

-Peripheral venous blood samples were collected aseptically into tubes containing EDTA from the enrolled patients with DM1, as well as control subjects. The peripheral blood mononuclear cells (PBMCs) were separated by density-gradient centrifugation **[Bøyum, 1976]** on Ficoll- Hypaque solution [Biotest, Germany]. The cell count was adjusted (after determination of cell viability via trypan blue dye; 0.4%, Sigma Co, USA) to 1×10^6 cells/ml of phosphate buffered saline (PBS) using Neubauer haemocytometer.

-For determination of the percentage of $CD4^+CD25^+$ T cells, and $CD4^+CD25^{hi}$ Tregs in the PBMCs of each sample; dual staining of the PBMCs was performed according to the manufacturer's instructions [BioLegend, San Diego, CA, USA] using fluorescein isothiocyanate (FITC)-conjugated anti-CD4, and phycoerythrin (PE)-conjugated anti-CD25 monoclonal antibodies. The $CD4^+CD25^{hi}$ Tregs were defined by gating on the highest 2-3% of the $CD4^+CD25^+$ T cells.

-To examine the expression of FOXP3; PBMCs of each sample were stained with PE-conjugated anti-FOXP3 monoclonal antibodies after their fixation and permealization according to the manufacturer's instructions [BioLegend, San Diego, CA, USA].

- For detection of non-antigen specific antibody binding; staining of the PBMCs with isotype and fluorochrome-matched control antibodies (IgG FITC-IgG2 PE) was conducted according to the manufacturer's instructions [BioLegend, San Diego, CA, USA] in parallel to the test tubes.
-The percentage of $CD4^{+}CD25^{hi}FOXP3^{+}$ Tregs was determined using fluorescent activated cell sorting (FACSCalibur) flow cytometer [Becton Dickinson, Sunnyval, CA, USA]. A minimum of 100,000 events were acquired and analyzed with CellQuest software [Becton Dickinson, Sunnyval, CA, USA].

Statistical analyses:-
All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) version 22.0 for Windows. Data were expressed as mean ± standard deviation (SD). Independent-sample t-tests were used to compare means between 2 groups. The one-way analysis of variance (ANOVA) test was used to compare means between diabetic patients and control subjects. A p value of < 0.05 was considered to be statistically significant.

Results:-

Clinical and laboratory characteristics of the study participants:-
The baseline characteristics of the study group are listed in table 1. No significant difference was detected between diabetic patients (including patients with new onset DM1 as well as those with established disease) and control group by means of age ($\boldsymbol{p} = 0.5$), gender ($\boldsymbol{p} = 0.5$), and BMI ($\boldsymbol{p} = 0.3$). On the other hand, patients with DM1 had significantly higher levels of HbA1c ($\boldsymbol{p} = 0.01$) in comparison to the age- and gender-matched healthy subjects from the control group. The high levels of HbA1c in the enrolled patients denote a poor glycaemic control, thereby increasing the risk of metabolic complications of DM1.

Frequency of $CD4^{+}CD25^{hi}FOXP3^{+}$ Tregs in the study group:-
Peripheral blood specimens from patients with DM1 and control group were analyzed with regard to the frequency of $CD4^{+}CD25^{hi}$ Tregs expressing FOXP3 transcription factor. Patients with recent onset DM1 had reduced frequency of circulating $CD4^{+}CD25^{hi}FOXP3^{+}$ Tregs when compared to patients with established disease [0.87 ± 0.11% *versus* 1.39 ± 0.41%, $\boldsymbol{p} = 0.02$] and to control subjects [0.87 ± 0.11% *versus* 2.91 ± 0.61%, $\boldsymbol{p} = 0.0001$]. On the other hand, no statistically significant difference was detected between patients with established disease and control subjects [$\boldsymbol{p} > 0.05$].

Discussion:-

In the past 10 years, there has been a steadily increasing concern in a subpopulation of T cells known as $CD4^{+}CD25^{hi}FOXP3^{+}$ Tregs. These Tregs can control the activation of autoreactive T cells that have escaped from the thymus, so they can prevent the development of autoimmune diseases, including DM1 **[Bisikirska and Herold, 2005]**.

The fundamental role of Tregs in autoimmune disorders, such as DM1, is well established. Tregs produce interleukin-10 (IL-10) resulting into down-regulation of inflammatory cytokines [interferon-γ (IFN-γ), and IL-17] and decreased expansion of effector cell pools. But, during progression of DM1, loss of FOXP3 expression occurs secondary to inflammatory signals. These, so called ex-FOXP3 Tregs gain an effector cell phenotype in terms of transcription factor expression and inflammatory cytokine production leading to DM1 pathogenesis **[Zhou et al., 2009]**.

In the present work, patients having recent onset DM1 showed reduced percentage of circulating $CD4^{+}CD25^{hi}FOXP3^{+}$ Tregs in comparison to patients with established disease [0.87 ± 0.11% *versus* 1.39 ± 0.41%, $\boldsymbol{p} = 0.02$], and to control subjects [0.87 ± 0.11% *versus* 2.91 ± 0.61%, $\boldsymbol{p} = 0.0001$]. On the contrary, no statistically significant difference was found between patients with established DM1 and control subjects [$\boldsymbol{p} > 0.05$].
Incongruent body of findings on the frequency of $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs in DM1 have been reported. The results of the present study come in agreement with those published by another group, where Ryba-Stanisławowska and associates found a decreased percentage of $CD4^{+}CD25^{hi}FOXP3^{+}$ Tregs in the peripheral blood samples of 47 type 1 diabetic patients in comparison to 28 healthy counterparts with a statistically significant difference ($\boldsymbol{p}$ =

0.0001) **[Ryba-Stanisławowska et al., 2014]**. In addition, Ryba-Stanisławowska et al. investigated the percentage of $CD4^+FOXP3^+$ Tregs among 36 patients with DM1, as well as 20 healthy control individuals. Similarly, the analysis of Tregs revealed lower percentage of $CD4^+FOXP3^+$ Tregs in type 1 diabetic patients as compared to healthy individuals from the control group with a statistical significance ($p = 0.0004$) **[Ryba-Stanisławowska et al., 2013]**. In a different study, He and his colleagues analysed the PBMCs from 35 children with DM1 as well as another 30 healthy children for the percentage of $CD4^+CD25^+FOXP3^+$ Tregs. Comparatively, a lower percentage of these cells was detected among diabetic children when compared to that in the control group ($p < 0.05$) **[He et al., 2014]**.
In contrast to the results of the aforementioned studies, Brusko and his group evaluated the percentage of $CD4^+CD25^{hi}FOXP3^+$ Tregs in 31 patients diagnosed with DM1, in addition to 33 control subjects. They concluded that no alterations in the frequency of peripheral blood $FOXP3^+$ Tregs in patients with DM1 *versus* healthy control subjects **[Brusko et al., 2007]**. Furthermore, Lawson and co-workers investigated the percentage of $CD4^+CD25^{hi}FOXP3^+$ Tregs in 44 patients with long standing DM1 (> 3 years) and 44 healthy control subjects. Noteworthy, they reported no difference in the percentage of $CD4^+CD25^{hi}$ cells co-expressing FOXP3 between patients with DM1 and healthy controls regardless of the level of CD25 expression **[Lawson et al., 2008]**. Likewise, Schneider and his collaborators executed another study on 13 patients diagnosed with DM1 and 18 healthy donors. They announced no difference in the proportion of $FOXP3^+$ Tregs among the $CD4^+CD25^{hi}$ cells between diabetic patients and control subjects **[Schneider et al., 2008]**.

The seeming divergence among different studies respecting the percentage of $CD4^+CD25^{hi}FOXP3^+$ in patients with DM1 could be traced to several reasons. **First**, although CD25 and FOXP3 represent convenient markers identifying a lineage of Tregs, there is a lack of a specific surface marker for Tregs hitherto; rendering their isolation difficult for phenotypic analysis. **Second**, different techniques for the isolation of Tregs have been adopted so far, mainly a paramagnetic beads-based procedure (MACS) and FACS. These methods, however, may yield different results. **Third**, most of the studies were performed on peripheral blood specimens, so may not reflect the proper T cell subsets present at the actual site of inflammation (i.e., the pancreas and pancreatic draining lymph nodes). **Finally**, the absence of appropriate matching for age in the healthy control population in some studies could also contribute to an evident inconsistency.

In conclusion, despite the conflicting results as regard to the frequencies of Tregs in type 1 diabetic patients; this study provides an evidence for a reduced frequency of $CD4^+CD25^{hi}FOXP3^+$ Tregs in patients with DM1, especially in those with disease of recent onset. Thus, these findings suggest a possible role for the quantitative deficiency of Tregs in the pathogenesis and development of DM1. However, other studies should be organized in the future for appraisal of the functional capacity of Tregs in patients with DM1, in order to tailor Tregs' therapy for this cohort of patients.

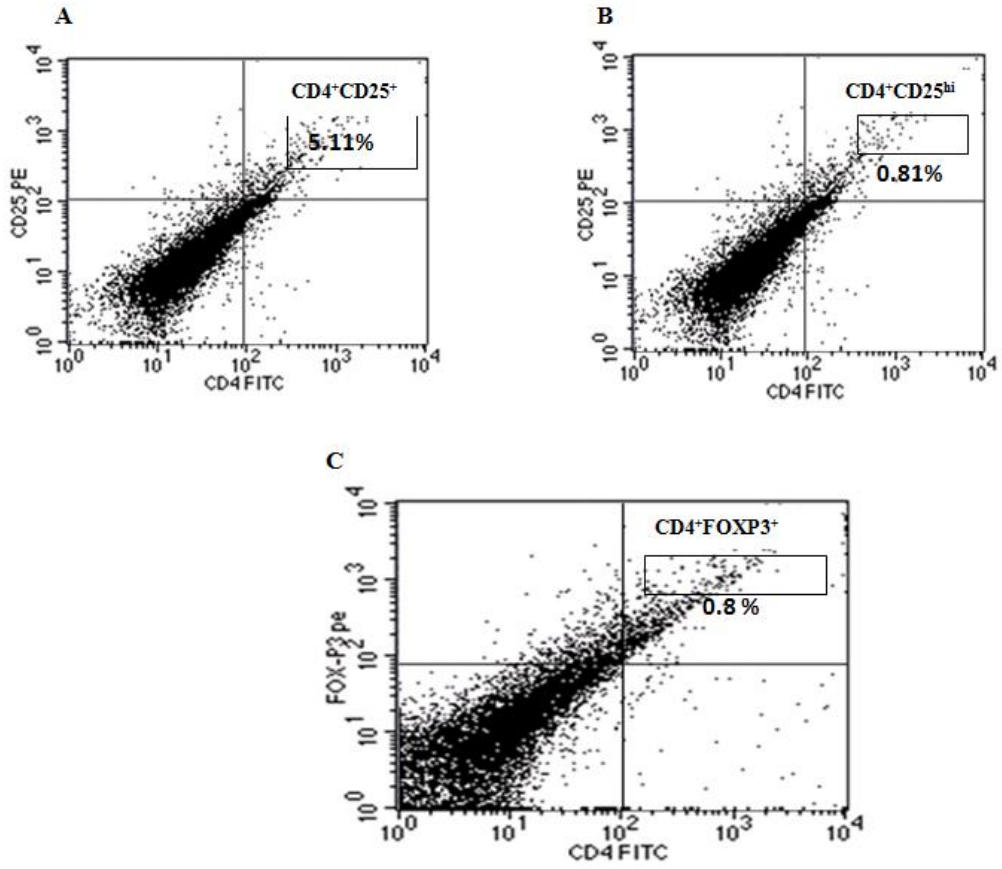

Fig (1): Representative flow cytometric dot plots from one patient with recent onset type 1 diabetes mellitus showing staining of the peripheral blood $CD4^+CD25^{hi}FOXP3^+$ Tregs. The PBMCs were stained with monoclonal antibodies against CD4, CD25, and FOXP3 molecules and analyzed using flow cytometry. The gate was set on $CD4^+CD25^+$ T cells based on the $CD4^+CD25^+$ gate [indicated by boxed region] **(A)**. Then, $CD4^+CD25^{hi}$ Tregs were defined by gating on the highest 2-3% of the $CD4^+CD25^+$ T cells [cells with a fluorescence intensity of CD25 exceeding 100 defined as bright] **(B)**. The cells were further gated based on FOXP3 expression and the percentage of $CD4^+CD25^{hi}FOXP3^+$ Tregs was determined **(C)**. $FOXP3^+$ Tregs predominantly exist within the $CD4^+CD25^+$ T cell quadrant. It is to be noted that, numbers within the upper right quadrants indicate percent of cellular events counted positive for the indicated markers **(A, B, C)**.

Table (1): Baseline characteristics of the study participants

Parameter	Group 1 (n= 17)	Group 2 (n= 15)	Group 3 (n= 24)	*p*-value*
Age (years)	20 ± 3	21 ± 1	19 ± 4.1	0.5
Gender (F/M)	10/7	9/6	13/11	0.5
BMI (kg/m^2)	20 ± 3.1	22 ± 0.9	22 ± 0.42	0.3
MBP (mmHg)	92 ± 11	93 ± 11	90 ± 12	0.4
FBG (mg/dL)	176 ± 54	118 ± 12	90 ± 10	**0.001**
HbA1c (%)	10.1 ± 2.3	8.5 ± 1.4	6.2 ± 1.1	**0.01**
Serum creatinine (mg/dL)	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.4

Legend: Data are presented as mean ± SD or number of patients. Group 1 (n= 17) represents patients with new onset type 1 diabetes mellitus [DM1], group 2 (n= 15) represents patients with established DM1, group 3 (n= 24) represents control subjects. **F/M:** female/male; **BMI:** body mass index; **MBP:** mean blood pressure; **FBG:** fasting blood glucose; **HbA1c:** glycosylated haemoglobin.

* One-way analysis of variance (ANOVA) test.

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Declaration of Interest:-
The authors declare no conflicts of interest.
Ethical approval:-
The protocol of this study was reviewed and approved by our institutional review board (R/16.05.89). All procedures were performed in accordance with the ethical standards of the institutional research committee and with Helsinki declaration.
Informed consent:-
Informed consent was obtained from all participants included in this study.

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