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ORGENAL ARTICLE

Detection of CD4+CD25+FOXP3+ T regulatory cells in Type 1 Diabetes Patients in Iraq by Flowcytometry analysis

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Abstract

Background: Regulatory T cells have an important role in the control of immune reactivity against self antigens and probably play a role in pathogenesis of type 1 diabetes (T1D). This study aimed to detect of regulatory T cells in type1 diabetes patients(T1D), and compared with healthy control subjects, and determination of GAD autoantibody.

Materials and Methods: 40 children and adolescents with T1D and 10 healthy subjects of matched age and sex as controls were enrolled in this study, at the AL-Kindy Specialized Center for Diabetes and Endocrinology Diabetic Center and the Central Child Hospital, their ages between (1- 18 year), are divided into three groups, newly diagnosed , chronic patients and healthy control groups. All cases were subjected to a thorough history taking, full clinical examinations and investigations which include; glutamic acid decarboxylase (GAD) autoantibody levels by using ELISA, and flow cytometric detection of regulatory T cells. **Results:** GAD autoantibody level was significantly higher in patient groups (newly diagnosed and chronic patients) compared with healthy controls. The percentages of CD4+CD25+Foxp3+ cells were significantly decreased in chronic patients of T1D than controls, while the percentages of CD4+CD25+ FOXP3+Treg were not significantly different between newly diagnosed patients and controls. Negative correlations were observed between GAD autoantibody and frequency of CD4+CD25+Foxp3. **Conclusion:** The percentage of regulatory T cells; CD4+CD25+Foxp3+ was decreased in patients with T1D and may have a role in its pathogenesis. Their role as a prognostic significance and their relation to various complications should be explored.

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which an inappropriate self-directed immune response affects and destroys insulin-producing beta-cells in pancreatic islets leading to dysregulated blood glucose levels. AlsoT1D is a T cell – mediated , chronic disease characterized by a deficient or absente of insulin , when the body´s own immune system attacks the β cells in the islets of langerhans of the pancreas (Andrea et al.,2013; Andras et al.,2013). The markers of the immune destruction of the beta cells include islet cell autoantibodies , autoantibodies to glutamic acid decarboxylase (GAD). These autoantibodies are present in 85-90% of individuals with type1 diabetes (ADA,2011). The glutamic acid decarboxylase (GAD) is a major autoantigen involved in the pathogenesis of type 1 diabetes mellitus, and GAD is one of the major antigens targeted by self reactive T-cells in T1D (Stina,2012; Yuichiro et al.,2012).

The peripheral tolerance to self antigens is maintained through several regulatory mechanisms including T regulatory cells (Asmaa et al.,2012; Weiting et al.,2013). Treg cells originally recognized by their constitutive expression of CD4+ and CD25+ further defined by expression of the transcription factor forkhead boxp3 FOXP3+ . (CD4+CD25+ Foxp3^{+}) regulatory T cells have a central role in protecting the individuals from autoimmunity by the critical contributors to its establishment and maintenance of immunological self – tolerance and the loss leads to T1D(Isabelle et al.,2014).

The aim of this study is to detection of T regulatory cells by the detection of CD4+CD25+FOXP3+ in peripheral blood of study groups using flow cytometery analysis, and determination of glutamic acid decarboxylase autoantibody (GAD) concentration in serum of study groups by using ELISA.

Material and Methods

Subjects and Methods:

Patients Group

This group included 66 subjects those were suffering from T1D after fasting blood glucose and HbA1c tests were done in a period between June and the end of November 2012 . They were selected from the AL-Kindy Specialized Center for Diabetes and Endocrinology and the Central Child Hospital. Their ages ranged from 1-18 years, with mean age of(7.7 ± 4.2 years)for short duration ≤ 3 months (newly diagnosed) group and mean age of (11.75 ± 4.19 years) for long duration >3 months (chronic) group.

Healthy control group

Ten healthy individuals with no family history of diabetes were considered as a control group. They were free from any systemic disease. Their ages ranged from 5-16 years with a mean age of (11.5 ± 6.3 years).

Laboratory analysis

Venous blood(3 ml) was withdrawn from each subject of the three groups . Some of blood put in EDTA tube used for flowcytometric analysis of human regulatory T cells using three fluorochrome-conjugated antibodies (using a kit supplied by R&D Systems ,U.S.A). The remaining blood was left to clot for 15 minutes, centrifuged and serum was separated some was used immediately for a glucose oxidase method of fasting blood glucose (FBG) analysis (supplied by GLU-PAP/United Kingdom),and measurement absorbance at 500nm by using spectrophotometer.

The remaining serum was kept frozen at –20°C for the subsequent assay of GAD autoantibody using by Enzyme Immunoassays for the quantitative determination of GAD Ab in human serum using a kit supplied by (RSR , United Kingdom) ELISA Kit .

Statistical analysis

Statistical Analysis System – SAS (2010) program was used to detect the effect of difference factors in study parameters. The linear relationship between variables was assessed by Pearson's correlation coefficient (r). For all tests, P values less than 0.05 were considered statistically significant .

Flow cytometric detection of regulatory T cells .

Wash peripheral blood mononuclear cells (PBMCs) (about 1x10^{6} cells per sample) with 2 mL of phosphate buffer saline (PBS) or Hanks' Balanced Salt Solution (HBSS), by spinning at 300 x g for 5 minutes, using 5 mL flow cytometry tubes. Remove all remaining PBS or HBSS and resuspend the samples in 100 µL of Flow cytometry staining buffer.

Add 10 µL of CD4-PerCP and 10 µL of CD25-PE antibodies or isotype controls (R&D Systems, Catalog # IC003P and IC003C). Incubate the mixture for 30 - 45 minutes at 2 - 8° C in the dark. Following the incubation, remove any excess antibody by washing the cells with 1 mL of Flow Cytometry FoxP3 Staining Buffer. After decanting the flow cytometry FoxP3 staining buffer, add 10 µL of FoxP3-APC antibody or goat IgG-APC isotype control to the samples in the remaining small volume of buffer (about 100 µL).Incubate the mixture for 1 hour at 2 - 8° C in the dark. Following the incubation, remove any excess antibody by washing the cells with 1 mL of Flow Cytometry FoxP3 Staining Buffer. The final cell pellet is resuspended in 200 - 400 µL of Flow Cytometry Staining Buffer for flow cytometric analysis .

Forward and side scatter histogram was used to define the lymphocyte population CD4 $^{+}$ CD25 $^{+}$ Foxp3+ regulatory T cells was evaluated as a percentage as shown in(Figure 2).

Results

Table (1) figure (1) showed the mean ± SE of glutamic acid decarboxylase autoantibody concentration for the study groups .There was a highly significant differences between patient groups (newly diagnosed and chronic patients) (158.75 ± 34.4 ; 176.58 ± 48.16) respectively, when compared with healthy control (3.87 ± 0.18) . In figure (1) demonstrated the highest concentration of GAD autoantibody in chronic patients group when compared with healthy control group according to cut off point negative < 5 ≥ positive.

Table 1 :- The concentration of GAD-autoantibody in serum of study groups of T1D and healthy subjects.

Study groups	N	%	CONCENTRATION U/ml Mean ± SE
Healthy control	10	20.0%	3.87 ± 0.18^{b}
Newly diagnosed patients	20	40.0%	158.75 ± 34.4^{a}
Chronic patients	20	40.0%	176.58 ± 48.16^{a}
Total	50	100	128.24 ± 23.22

(a,b) Small letters denoted that is high Significant differences between groups of study at (p≤ 0.05). Assay Cut off, negative < 5≥positive .

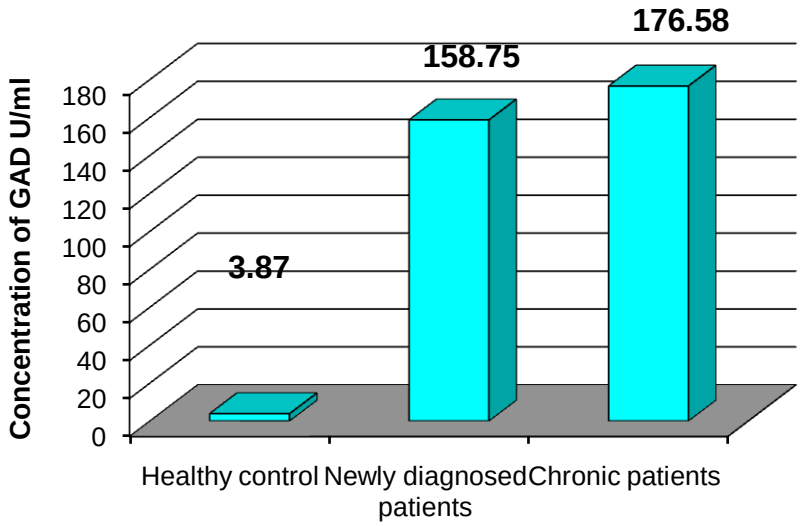

Figure 1: Concentration of GAD –autoantibody in serum of study groups

Table 2 and figure 2 showed a significant increase in CD4+and CD25+ in newly diagnosed patients (56.84±3.77and 54.74±3.17) respectively . No significant differences in CD25+ between newly diagnosed patients and healthy control, while there was a decrease in CD4+ and CD25+ percentage in chronic patients. The percentage of CD4+FOXP3+ and CD25+FOXP3+ was significantly increased in newly diagnosed patients (7.80 ± 0.95and 7.57±0.89) respectively compared with chronic patients. Also a significant decrease was found in the above parameters of chronic patients when compared with newly diagnosed.

Table 2:- Detection of $CD4^+$ $CD25^+FOXP^+3$ in study groups blood of T1D using flowcytometry.

GROUPS	N	$CD4^+$ % Mean ± SE	$CD25^+$ % Mean ± SE	$CD\ 4^+FOXP3^+$ % Mean ± SE	$CD25^+FOXP3^+$ % Mean ± SE
Healthy control	10	51.17 ± 3.00^b	52.22 ± 3.20^a	7.57 ± 0.68^a	7.57 ± 0.71^a
Newly diagnosed patients	20	56.84 ± 3.77^a	54.74 ± 3.17^a	7.80 ± 0.95^a	7.57 ± 0.89^a
Chronic patients	20	40.46 ± 2.95^c	36.96 ± 2.55^b	2.53 ± 0.31^b	2.48 ± 0.29^b
Total	50	49.15± 2.23	47.12± 2.09	5.65 ± 0.55	5.53 ± 0.53

(a,b,c) Small different letters denoted that high significant between CD markers at ($p \leq 0.05$).

Table 3 explained the correlation between GAD and other study parameters. The results showed a negative correlation between GAD and CD4+ , CD4+FOXP3+, CD25+, CD25+FOXP3+ (r=- 0.382,- 0.307,- 0.350, -0.308) respectively**.**

Table 3:- Correlation coefficient (r) between GAD- autoantibody with other Parameters.

Marker / Parameters	GAD	Level of Sig.
$CD4^+$	0.382-	**
$CD4^+FOXP3^+$	0.307 -	*
$CD25^+$	0 .350 -	*
$CD25^+FOXP3^+$	0.308-	*

******Correlation is significant at($p< 0.01$) ,***** Correlation is significant at ($p<0.05$).GAD: Glutamic acid decarboxylase .

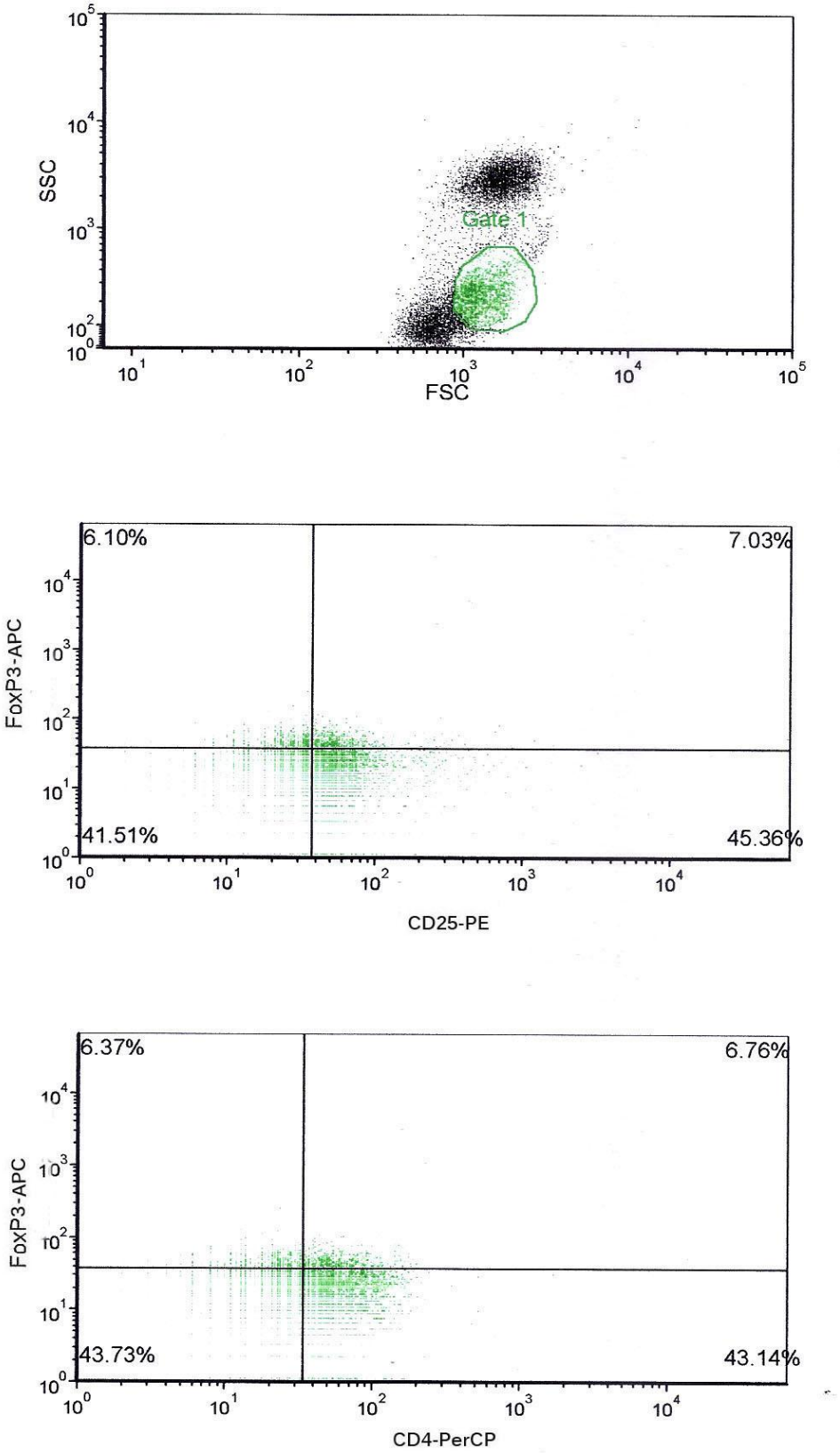
SSC
FSC
Gate 1
6.10%
7.03%
41.51%
45.36%
FoxP3-APC
CD25-PE
6.37%
6.76%
43.73%
43.14%
FoxP3-APC
CD4-PerCP

Figure 2.A

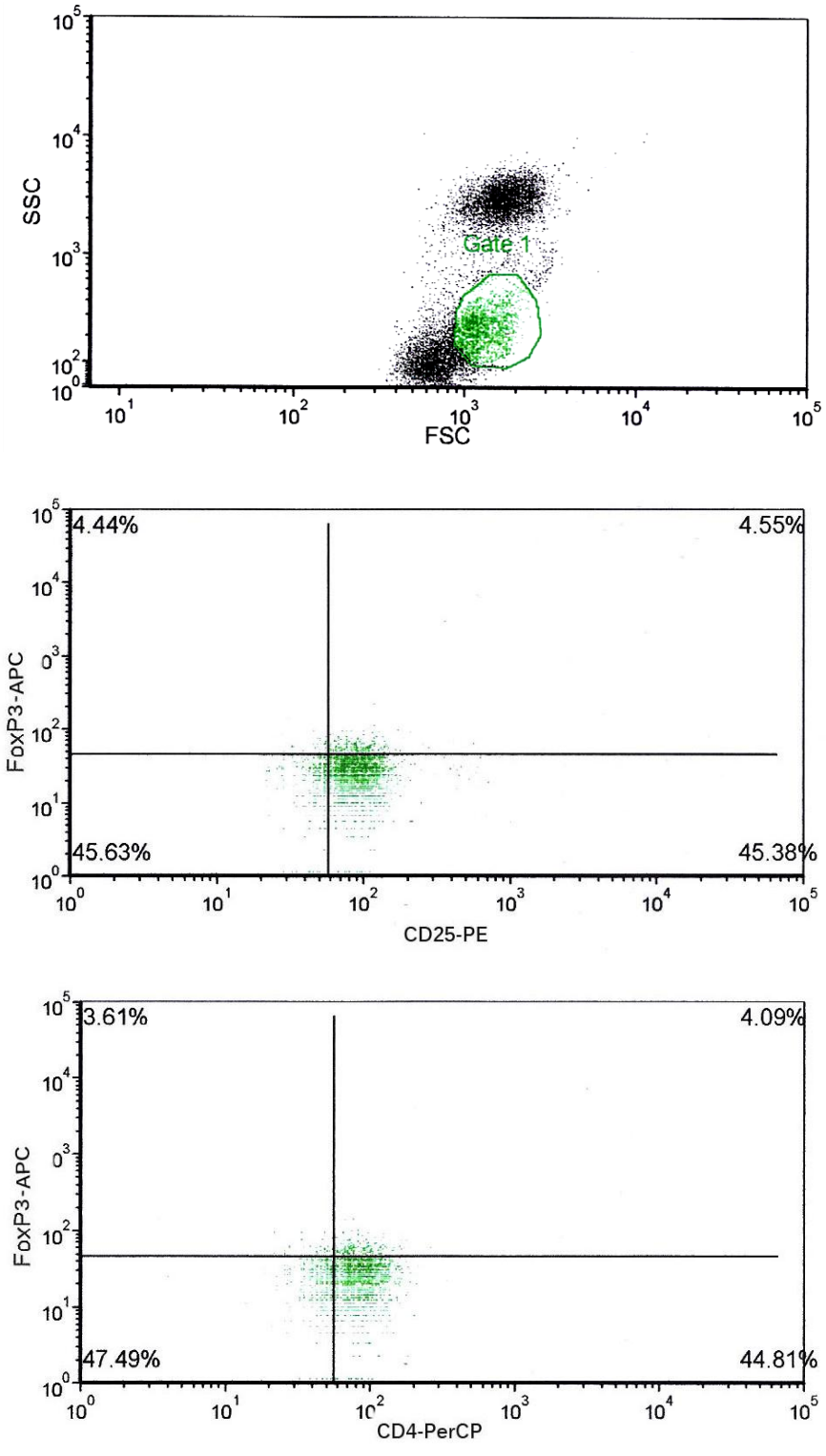
SSC
FSC
Gate 1
FoxP3-APC
CD25-PE
4.44%
4.55%
45.63%
45.38%
FoxP3-APC
CD4-PerCP
3.61%
4.09%
47.49%
44.81%

Figure 2.B

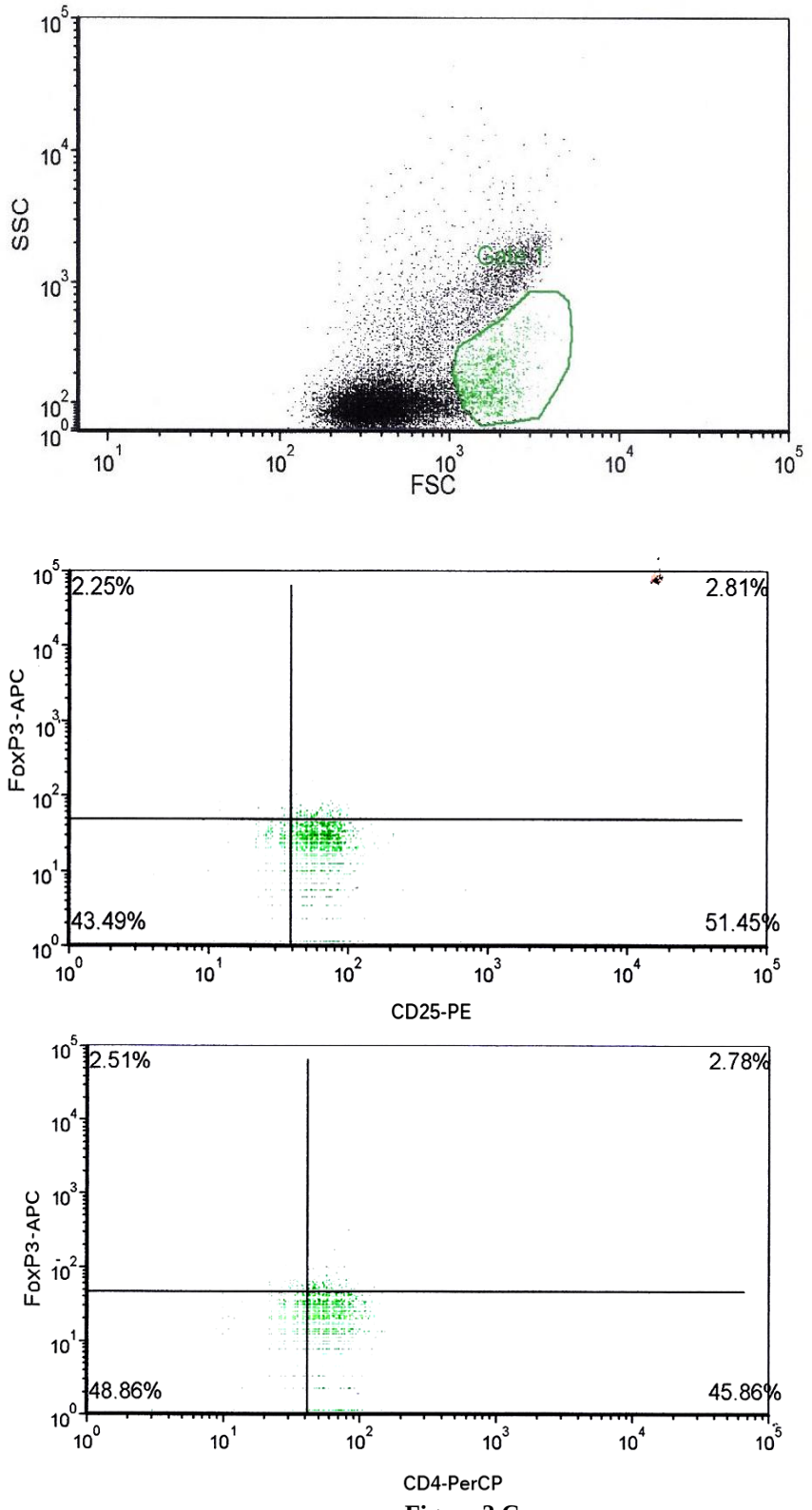

Figure 2.C

Figure (2.A.B.C):- PBMCs were harvested and stained ,The percentage of CD4+,CD25+,FOXP3+ in some patients groups using flowcytometry. Lymphocyte population was gated based on forward and side scatter, the gate were used for analysis . Dot plots show the relative CD4+,CD25+, and FOXP3 populations. (A) The

correlation between CD4+,CD25+ expression and intracellular FOXP3+ expression in healthy controls. (B) The correlation between CD4+,CD25+ expression and intracellular FOXP3+ expression in newly diagnosed patients. (C) The correlation between CD4+,CD25+ expression and intracellular FOXP3+ expression in chronic patients.

Discussion

This is a first study on detection of regulatory T-cells (T-reg) subpopulations CD4+CD25+FOXP3+ by flowcytometric analysis in blood of type1 diabetic patients in Iraq. Treg cells defined by the expression of CD4+CD25+ and the transcription factor forkhead boxP3(FOXP3+) which play a critical role in the maintenance of peripheral tolerance and in the control of autoimmunity (Jean et al.,2009 and Jane,2010;).Impairment of Treg development and/or function can precipitate a variety of autoimmune diseases, whereas a higher frequency of Tregs can render the immune system hyporesponsive to pathogens .The results observed marked decrease in Treg percentage in patients of T1D compared with healthy controls. This is in agreement with Anna ,(2011); Monika et al.,(2011);Zhou et al.,(2011); Qin et al.,(2011); Agnieszka et al.,(2012); Marek-Trzonkowska et al., (2012) Dalia et al.,(2013) who found a defect in immune regulation which was expressed by a lower percentage of CD4+CD25+FOXP3+T regs found in the pathogensis of diabetes in man. The present results confirmed previous studies of Gao et al.,(2012);Robert et al.,(2012). Who found that FOXP3+ is critical for the development and function of Treg cells , and is a signature transcription factor for Tregs which have a central role supporting peripheral tolerance towards the body s̀ own antigens .Our observation of decreased FOXP3 in diabetic patients, suggests that the mechanisms mediating the induction and maintenance of peripheral tolerance may be disrupted by the cytokine response associated with destruction in the pancreas. Whereas, our results were in disagreement with previous studies of EL-Araby,(2012); Sundararajan et al.,(2012);Elena et al.,(2013) Who they reported that there was no difference in the number of CD4+CD25+Tcells between type1 diabetes patients and normal controls. The quantitative defects of Treg cells may be due to the chronic inflammation which was observed in diabetic type1 patients ,so the number and/or function of Tregs were impaired not able to prevent the activity of inflammatory cells .Also because T1D is a T-cell mediated autoimmune disorder ,death of Treg cells that control proliferation of effector T- cells could contribute to the pathogensis of diabetes as well as provide a sign for autoimmunity. Also, the identification of Foxp3 as a regulatory lineage specific factor provided a useful phenotypic and optimal marker for regulatory T cells, and the suppressive phenotype and the development of regulatory function depend on the expression of Foxp3.Indeed, recent results indicate that Foxp3 behaves as a master regulator of the regulatory T cells phenotype (Sakaguchi,2008). The American Diabetes Association defined patients with type1 diabetes as those who have an immunologic disorder such as GAD antibody positive Hui et al.,(2013). Which may be used as an earlier marker than FBG and are very robust tools for predication of developing of T1D . Hassan,(2008)who studied of the Iraqi patients who found the seropositivity for GAD antibody was high, which indicated the progressive nature of the autoantibody stimulation which seems to be in consistency with the level of β- cell destruction . In comparison a high GAD seropositivity was reported for T1D patients group in this study which verifies the suspected role played by this antibody in the pathogenesis of the disease, and was similar to Iraqi study of Madha et al.,(2012) who reported that GAD antibody in patients was high. Whereas, it disagreed with Sergio and Marilia,(2009) who explained that the presence of these autoantibody for at least 3 to 6 months indicate the presence of autoimmunity against pancreatic islets, In type 1 diabetes, the immunological tolerance to islet autoantigens has been lose ,and the processing of islet proteins was presented by antigen-presenting cells (APC) to islet autoreactive T cells, leading to a breakdown in the tolerance. Additional islet autoreactive T cells may become activated (epitope spreading), which may contribute to β-cell destruction . CD4 T cells activate B cells to produce islet autoantibodies. Presentation of islet autoantigen to the immune system may also lead to activation of regulatory T cells (Tregs) leading to inhibition of proinflammtory islet autoimmunity. Leading to diminished numbers of CD4+CD25+FOXP3+ Treg cells in patients with type1 diabetes . In addition to that autoantibody were produced in type 1 diabetic patients , thus there was inverse significant correlation between GAD and other immunological parameters.This results were in agreement with Roep and Peakman(2012), who found in Finland study that CD4+T cells recognizing the GAD 65 peptide. In addition increased CD4+ responses to the same GAD epitope were observed in children at risk and diabetic. Whereas our results were disagreed with Lindley et al.,(2005) and Chao et al.,(2013).The first authors observed no significant correlation between T reg cells to GAD in insulin- dependent diabetes, and the second, showed a weak relation with clinical features after the diagnosis of T1D.

Conclusions

This study concluded that patients with T1D have lower percentages of CD4+CD25+FOXP3+ regulatory T cells in the peripheral blood which correlated negatively with GAD autoantibody ,and high concentration of GAD autoantibody was found in the most of T1D patients and is a very robust tools for predication of developing of T1D.

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