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Foxp3+ and CD4CD25+ T cells and its Clinical Relation to Type 1 Diabetes

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Key Words: T1D, Foxp3, Tregs and CD4+CD25+ T cells

1. Introduction:

1.1. Regulatory CD4+CD25+ T cells " T regulatory (Tr) cells are a distinct population of T cells which modulate T helper Th1 and Th2 mediated immuno-responses and maintain immunological homeostasis." Therefore, there are two subsets of T reg Tr type 1(Tr1) and CD4CD25 T lymphocytes the well described subsets of CD4+ Tr cells. CD4+CD25+ T lymphocytes are naturally occurring CD4 T lymphocytes" that express CD marker number 25 that arises from thymus and seeds into periphery, creating a cohort of cells with profound T-cells immunesuppressive qualities." Cells that does not express CD4CD25 (CD4-CD25-) T-lymphocytes are detected in human blood and can suppress proliferation and production of cytokines from T lymphocytes that does not express CD4CD4 (CD4-CD4-)T lymphocyte in vitro in which a cell could not attached to another one in a contact dependent manner (Jonuleit et. al,2001; Dieckmann et. al,2001; Levings et. al.,2001; Ng et. al.,2001 and Piccirillo et.al. 2001).

Tr1 and CD4+CD25+ T lymphocytes are similar to each other in biological characteristics like slow or absence of cell division in vitro, and suppress capacity in vivo and in vitro. Activity of Tr1 subsets is by secretion of "IL-10 and (TGF-β)", while suppressive mechanism of CD4+CD25+ T lymphocytes have not well explained. CD4+CD25+ T- lymphocytes have been largely studied in autoimmune diseases, whereas Tr1 cells have been widely investigated in transplantation, chronic infectious diseases and allergy. Many other researchers are performed to understand the correlation between Tr cells groups. More studies made a hope for treatment and therapy to prevent and modulate T-cell mediated disorders (Richard et.al., 2004). Regulatory T lymphocytes (CD4CD25) Tregs have a close relation in suppression of immunity(Hori et.al.,2004; Ramsdell ,2003 and Asseman and von Herrath,2002). Many other studies using experimental animals and human studies suggested that Treg cells may have a crucial role in the initiation of autoimmune disorders by decreasing or diminishing functions (Baecher-Allan et.al.,2004). Injection of Tergs into mice that susceptible to disease, prevents initiation of autoimmune disorders, while other strains of mice can develop gastritis, prostatitis and thyroiditis in the absence of C4CD25(Asano et. Al,1996; Sakaguchi et.al, 2001 and Takahashi 1998). **Foxp3** is the abbreviation of Forkhead (FKH) box protein 3belonging to a family of forkhead and have a crucial role in the production and function of Tregs that is essential for tolerance of autoimmunity to self. The main task of immune system is to keep a balance between killing foreign bodies that attack human body and prevents self molecules from immune mediated damage as a sequence to inflammation and autoimmune disorders. Tregs have a role in controlling the above balance by suppression of immune response. The most important type of Tregs is that express CD4+ and Foxp3 which is largely studied because of therapeutic properties in immune disorders (Allan et. At.,2008). While Treg suppress activities of CD4+CD4+ T- lymphocytes, B- lymphocytes and Antigen presenting cell (APC) not well explained. Development and function of Foxp3 in Tregs were fully defined by many different researchers (Miyara and Sakaguchi,2007; Chen et.al.,2008 and Zuo et.al.,2007).

1.1.2. Discovery Foxp3 and Key Structural Feature is discovered in 2001 as an X- linked gene in 3 separate researches, where the immune regulation importance was first suggested in scurfy mouse as a mutated gene by Brunkow et.al. in 2001and in Immune dysregulation, polyendocrinopathy, enteropathy and X- linked (IPEX) patients by Benett and wilden in 2001, the above mice have an X-linked gene spontaneous mutation "rapidly fatal autoimmune disorders which is mediated by CD4+ T-cells" (Godfrey et.al,1991).. The sequence of mutated genes was analyzed showed a protein have a standard domain of FKH and the same researchers recognize an unusual transcriptional factor related to FKH family(Godfrey et.al,1991). Other studies established that foxp3 can bind to the consensus sequence of FKH binding sites in the variable region of transthyretine and immunoglobulin variable region (V1P) promoter. It was suggested that foxp3 prevents transcription until it suppressed luciferase activity operated by SV40 promoter fixed to 3 consensus sequence of V1P forkhead, while others in their studies showed that foxp3 is able to be a transcriptional activator(Schubert et.al,2001).

1.1.3. Phenotype and function of foxp3 " In the immune system, foxp3 is expressed by a population of CD4+ Treg cells"(Khattri et al. 2003; Hori et,al,2003 and – Fontenot et.al,2003). The recognition of foxp3 depends mainly on great secretion of R α chain of IL-2 and activity and suppression of immunity in vitro and in vivo (Sakaguchi et.al,1995 &. Suri-Payer et.al.,1998). Several researchers depend on the CD25 expression to Tregs and study biological characteristics since "foxp3 protein is a nuclear live FOXP3+ cells cannot be isolated except from reporter mice;(Maynard et.al. ,2007; Lin et.al.,2007 ; Wan and Flavell, 2005)∀. Φοξπ3 Τρεγσ ηασε αν εσιδεντ οφ υνιθυε προπερτιεσ ισ τηε αβιλιτψ το προδυχε χψτοκινε τηατ ισορδιναρ ιλψ δεριϖεδ φρομ Τ λψμπηοχψτεσ λικε INF ⊥, IL-2 or tumor necrosis factor (TNF) α, and weak response to triggering of TCR in vitro. Still the ability to stimulate immune suppression cytokine is retained, like IL-35, TGF-β and IL-10(Levings ,2001; Dieckmann et.al,2001; Jonuleit ετ.αλ,2001; Thornton and Shevach,1998; Takahashi ετ.αλ,1998; Collison et al, 2007;

Niedbala et al.,2007; Sakaguchi et al.,2006; . Lan et al.,2005. Mays & Chen,2007 and Liu et al,2006).

1.1.4. Role of foxp3 in the Development of Tregs Cells. Naturally Occuring Versus Induced Treg Cells Some of Terg cells develop in the thymus others in periphery when virgin CD4 T-lymphocytes Ags in a tolerogenic conditions. Naturally occurring Treg (N-Treg) could not be differentiated from peripheral one (periphery induced Treg (I-Treg). Although antigenic specificity of Treg cell is variable, it prefers to recognize self (Lathrop et al.,2008), and are selected when the strength of TCR signalling is above that of classical positive selection but below that of negative selection.(Jordan et.al., 2001). On other hands I-Treg cells is specific for the presented Ags beside tolerogenic dendritic cells or cytokines that suppress immunity like TGF-β,(Kretschmer et al.,2005). Absence of foxp3 have a role in autoimmune disorders, so it is required for usual activity of Treg cells when the exact role of foxp3 in the differentiation of N-Treg cells in the thymus is still under discussion Fontento et.al. discovered in 2003 that mice that had not foxp3 gene in the stem cells could not develop foxp3 CD4+CD25+ T- lymphocytes[23] , so foxp3 is essential to this process. While in 2007 Lin and Ganum and their colleagues discovered that deficiency of foxp3 did not prevent development of cells that express "Treg- associated gene signature and cell surface molecules" where foxp3 is still needed in suppressive activity. Hence, the function of foxp3 is not only to determine lineage of Treg cells, but is to increase and fix predetermine Treg lineage. Hill suggested in 2007 that foxp3 does not control Treg cell signature straightly because it contains a group of genes , instead of transactivation foxp3 co-active these genes clustering.

1.1.5. Role of Foxp3 in Conventional T-Cells When human T- lymphocytes are activated, they express foxp3 transiently which is an incomparable characteristics of conventional CD4+ T-lymphocytes. Under strong activation and after 3 days of TCR stimulation all T effector (T eff) cells express foxp3 become foxp3+ve cells[32]. The basic difference between Treg cells and activated Teff cells stays apparent . Although the foxp3 genes expression of stimulated Teff cells reach that of other Treg cells, it still under than that of likewise stimulated Treg cells. Human CD4+ T- lymphocytes are able to change into Tregs only when the expression of foxp3 was increased and fixed[32,14]. While others found that initiation and activation of Treg cells fundamentally depends on degree and durance of foxp3 expression, but not on foxp3 existence or not. A constant fact that the transient expression of foxp3 in activated Teff cells is not enough to inhibit division of cells and production of cytokines[33,34,35]. There is an essential differences between Treg and Teff cells in foxp3 molecular activity such as a result of interactive with other proteins that is Treg-specific. Pillai et. al.,2007 and Walker et al.,2003 stated that suppressive activity of foxp3 Teff cells is not permanent(transient)[36,37]. On other hands, Morgan and other researchers could not indicate this truth (Morgan et al. ,2005; Allan et al.,2007 and Wang et al., 2007).

2. Materials and Methods:

2.1. Patients: Forty four patients children with newly diagnosed T1D admitted to AL-Mansour Hospital for Children and AL-Iskan Hospital for Children in Baghdad and 20 control healthy individuals were included in this study.

2.2.Methods:

2.3. Sample Collection :

Two milliliters of blood were collected in EDTA tubes and were transmitted within 45 minutes to the laboratory where CD4CD25, and Foxp3 are tested.

2.4. Isolation of Lymphocytes:

Whole fresh blood samples in EDTA were diluted with phosphate buffer saline (PBS) in a ratio 1:1. Lymphocytes were separated using Ficoll by adding 2 ml of diluted blood into 2 ml of Ficoll. Diluted blood should be added carefully at the wall of centrifuge tube, then tubes were centrifuged at 2500 rpm for 30 minutes. Lymphocytes were separated depending on density gradient of lymphoprep centrifugation, lymphocytes were isolated by sucking using Pasteur pipette then were washed in Phosphate Buffer Saline (PBS) in experiments of CD4CD25 and Foxp3. Cell concentrations were adjusted to approximately $2x10^5$ cell/ml in the buffer. Cells are checked for viability by trypan blue exclusion using cell count chamber.

2.5. Estimation of CD4CD25 T cells in Blood of Patients with T1D by Flowcytometry Technique :

Applications Tested This cocktail has been pre-titrated and tested by flow cytometric analysis of normal human peripheral blood cells . This can be used at 20 µL per test (containing 1.0 µg of Anti-Human CD4 FITC) and 0.125 µg of Anti-Human CD25 (BC96) PE) . A test is defined as the amount (µg) of antibody that will stain a cell sample in a final volume of 100 µL. Cell number was determined from 105 to 108 cells/test.

2.6. Estimation of Foxp3+ T cells in Blood patients with T1D: This 236A/E7 antibody has been pre-titrated and tested by intracellular staining and flow cytometric analysis of normal human peripheral blood cells using the Foxp3/Transcription Factor Buffer Set (cat. 00-5523) and protocol. (nuclear) intracellular proteins. This can be used at 5 µL (0.25 µg) per test. A test is defined as the amount (µg) of antibody that will stain a cell sample in a final volume of 100 µL. Cell number should be determined empirically but can range from 10^5 to 10^8 cells/test.

3. Results and discussion

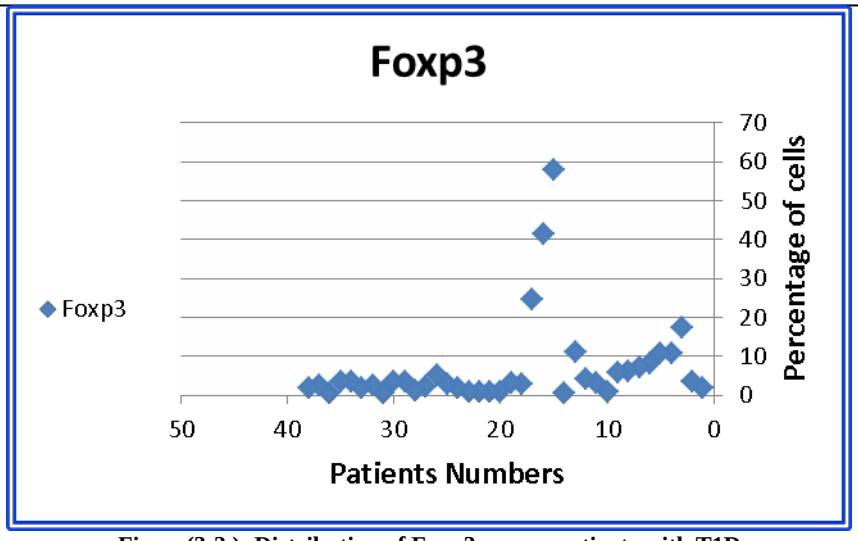
3.1.Flowcytometry Experiments

Flowcytometry was done to 45 (56.2%) of the patients, but was not done for the rest of the samples 35 (43.8%), because it was not possible to get whole blood samples from the patients, either because refusal of their families or difficulties of blood withdrawn because of their ages.

3.2. Tregs (CD4+CD25+, CD4+.CD25+ and Foxp3 T cells:

3.2.1. Foxp3+ T cells

A significant differences were obtained between levels of Foxp3 of patients and control P value= 0.0001 as shown in table (3-6). The range of Foxp3 was (0.6% - 59%) while control was 1% - 3% mean level of patients was 7.15, SE ±0.5.



Figure(3-3): Distribution of Foxp3 among patients with T1D

Table (3-6): Mean and standard deviation of the studied parameter among control and diabetic

	Control	Diabetic	P-value
CD4	0.41 ±0.28	1.42 ±1.85	0
CD25	0.25 ±0.15	2.24 ±1.21	0
CD4CD25	0.26 ±0.20	1.30 ±1.04	0
Foxp3	1.86 ±0.59	2.64 ±1.79	0.037
Anx V	0.06 ±0.04	2.81 ±2.75	0

Highly Significant at level P < 0.01

Table 3-6 shows that there is a highly significant differences among all the studied parameters by using ANOVA test.

Table (3-7): Mean and standard deviation of the studied parameter among control and diabetic

	Control	Diabetic	P-value
CD4	0.41 ±0.28	1.42 ±1.85	0
CD25	0.25 ±0.18	2.24 ±1.21	0
CD4CD25	0.26 ±0.2	1.3 ±1.04	0
Foxp3	1.86 ±.59	2.64 ±1.79	0.001
Anx V	.06 ±.03	2.81 ±2.45	0.002

Highly Significant at level P < 0.01

Table 3-7 shows that there is a highly significant differences among all the studied parameters by using ANOVA test. Mean results of CD4 ±SD in control was 0.41 ±0.28 but the result of CD4 ± SD in diabetic patients showed an elevation to 1.4 ± 1.85 which means that diabetes has an impact on patients represented by this increase in concentration.

Table (3-8): Mean and standard deviation of the studied parameter among control and diabetic

	Control	Diabetic	P-value
CD4	0.41 ±0.28	1.42 ±1.85	0.01
CD25	0.25 ±0.15	2.24 ±1.21	0.001
CD4CD25	0.26 ±0.20	1.30 ±1.04	0.018
Foxp3	1.86 ±0.59	2.64 ±1.79	0.267
Anx V	0.06 ±0.04	2.81 ±2.75	0

Highly Significant at level P < 0.01

Table 3-8 shows that there is a highly significant differences among all the studied parameters except for Foxp3by using ANOVA test.

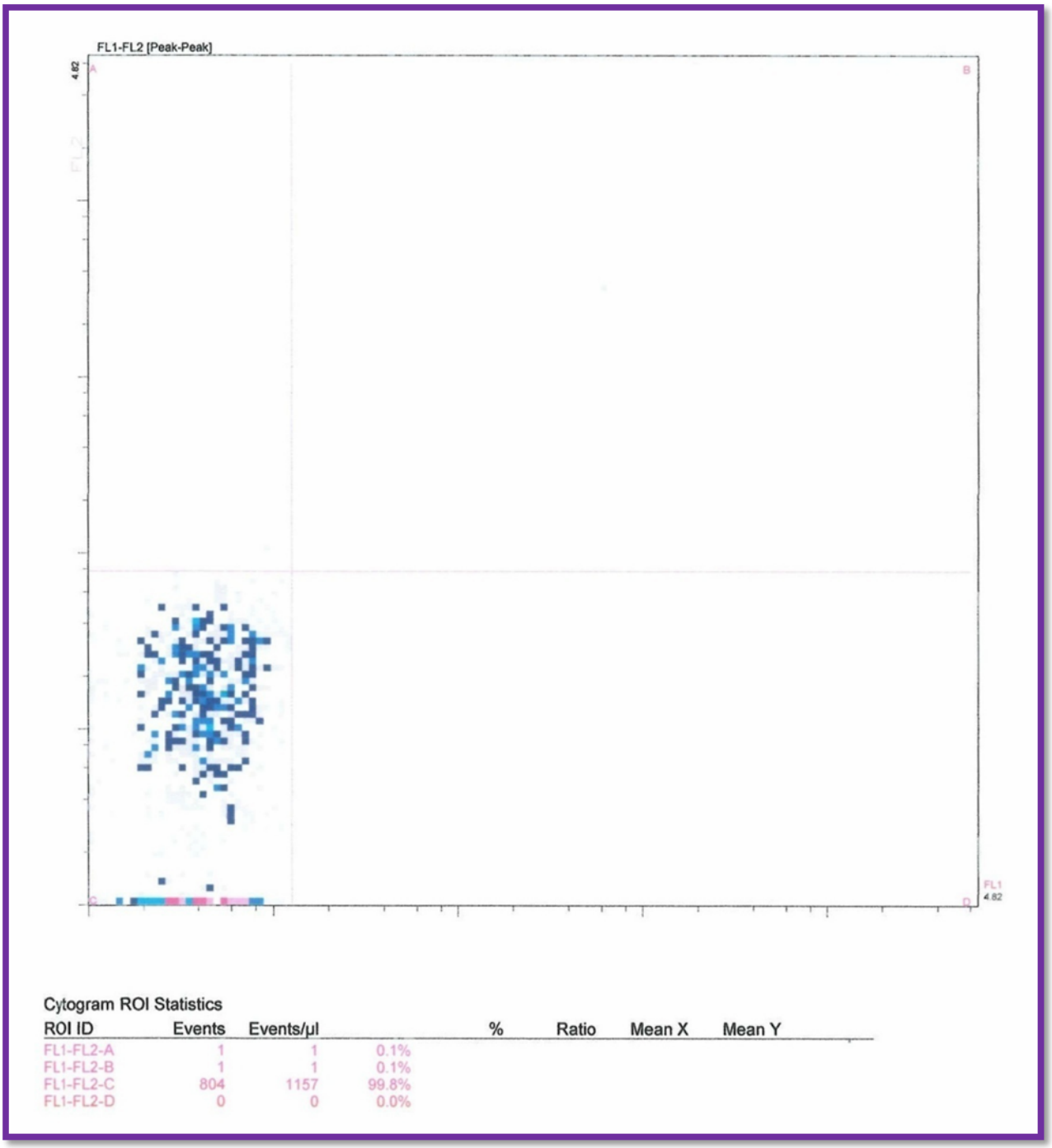
Table 3-7 shows that there is a highly significant differences among all the studied parameters by using ANOVA test. Mean results of CD4 ±SD in control was 0.41±0.28 0.25±0.18, 0.26±0.2, 1.86±.59 and .06±03 respectively, but the result of CD4,CD25, CD4CD25,Foxp3 and Anx V± SD in diabetic patients showed an elevation to 1.4 ± 1.85, 2.24±1.21, 1.3±1.04, 2.64±1.79 and 2.81±2.45 respectively, which means that diabetes has an impact on patients represented by this increase in concentration.

Different researchers proved the effect of other cell markers in initiation or protection of T1D like the two subset of Th cells and their cytokines , Jailwala and his colleagues in 2009 stated that destruction B cells and the breakdown of self tolerance of immunity requires not only genetic predisposition and environmental effect but also needs balance in number and function between killer (e.g., CD4+ and CD8+ effectors) and regulatory T-cells in the pancreatic infiltrate, CD8+ T cells are important to initiate β cell injury leading to the activation amplification of CD4+ T cells(Jailwala et.al.,2009).
In an experiment on mice Che and his colleagues found that INF produced during viral infection may have a role in maintaining foxp3 expression and activity of T regulatory cells(Che et.al.,2015). According to tables below 3-9,3-10 ,3-11and 3-12 frequency of CD4+CD25+ Foxp3 cells was positively correlated with age of the patients (r=0.585 p=0.00) (Assama et.al.,).

Table(3-9): Ranges and means of Foxp3 levels for each age group

No. of Group	Age Ranges	No. of Patients(%)	No. of male(%)	No. of female(%)	Foxp3 ranges	Foxp3 means
1	0-3	4(8.88)	0(0)	4(8.88)	2.2-2.5	2.4
2	4-7	4(8.88)	2(4.44)	2(4.44)	0.4-57.7	19.56
3	8-11	24(53.33)	8(17.77)	16(35.55)	0.7-41.4	10.1
4	12-15	10(22.22)	4(8.88)	6(13.33)	0.7-17.3	3.78
5	16-19	3(6.66)	1(2.22)	2(4.44)	1.7-5.2	3.45

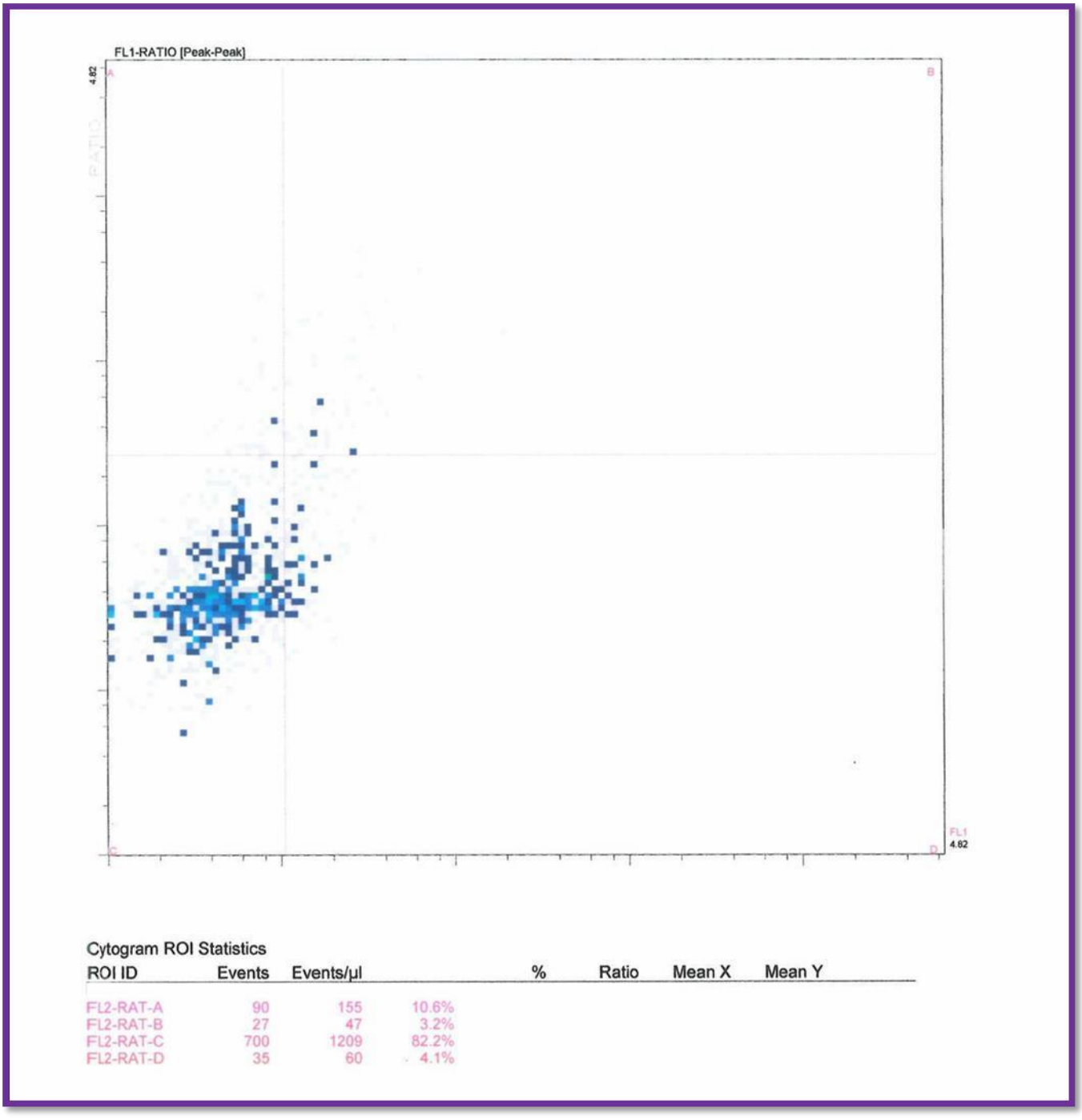
The above table showed age groups of children included in flowcytometry study according to levels of Foxp3, in comparison with figure 3-2 peak age incidence was 10 years for female and 12 years for male in the above table peak age group was 8-11 for female(35.55%) and (17.77%) for male of patients included in flowcytometry study. Mean values of Foxp3 for all groups were 2.4, 19.56, 10.1, 3.78, and 3.45 for age groups of 0-3, 4-7, 8-11, 12-15 and 16-19, respectively. The age groups 4-7 and 8-11 revealed high mean of Foxp3 levels 19.56 and 10.1 respectively, these high mean values because of three patients in these groups had high levels of Foxp3 57.7, 41.4 and 24.5, but when they were neglected or ignored the means of Foxp3 of these two groups became 0.9 ,3.34 respectively. See Figures 3-4,3-5,3-6, 3-7, 3-8 and 3-9. A study of Gregg et.al.,2005 investigated CD4+CD25+ T cells in normal healthy individuals, the researchers demonstrated that these cells increases with age. The explanation of this increase in relation to immune response remains unclear. Simone et.al.,2008 reported that CD8+ T cells increased with age.



Cytogram ROI Statistics

ROI ID	Events	Events/µl	%	Ratio	Mean X	Mean Y
FL1-FL2-A	1	1	0.1%			
FL1-FL2-B	1	1	0.1%			
FL1-FL2-C	804	1157	99.8%			
FL1-FL2-D	0	0	0.0%			

Figure (3-4): Test control of Foxp3, the value was 0.0%



Figure(3-8): The approximate mean level of Foxp3 of group number 4 and 5

CD4+CD25+ T cells

In this study table 3-6 shows that mean and standard deviation of CD4+CD25+ T cels in control is 0.26±0.2 increased in diabetics to 1.3±1.04 with further increase into 10.26±9.87 and the p value by applying

Anova test is 0.000. In discussing these results we should bear in mind that patients involved in this study were in the early stage of disease and infection, so the high percentage of CD4+CD25+ T cells is related to the early stage of the disease and with time the function and not the number will be decreased with the consequences of susceptibility to autoimmune disease and loss of tolerance.

Lindely et.al.,2005 stated that although there was a high number of CD4+CD25+ T cells in the host, these hosts have a high diabetes because of the functional deficiency in their regulatory functions which lead to the lack of self tolerance evident in patients with T1D.

Kukreja et al., 2005 showed that CD4+ T cells secreting INF-$^{\gamma}$ (Th1) was significantly reduced in newly diagnosed to control long standing in autoimmune-mediating diabetes compared to control. IL-4(Th2) producing cells showed no reduction in number compared to controls, so showed that there was a deficiency in immunoregulatory cells of CD4+ T cells in newly diagnosed T1D.

Finally they suggested that islets cells autoimmunity is caused by a multiple immunoregulatory T (Tregs) function defects such as decreasing secretion of INF-$^{\gamma}$ and IL-4.

Similarly, in other study reported that a significant difference was observed in Foxp3 T cell% cystic fibrosis (CF) : (60.7 ± 6.19) versus controls (76.8 ± 5.16), $p < 0.001$, i.e. there is a decreased levels in $CD4^{+}CD25^{high}$ $Foxp3^{+}$ T cells in children with CF". (Anil and Singha, 2015). Results of the present study stated a significant increase in the levels of CD4+CD25+ and Foxp3in children with T1D, whereas in systemic lupus erythematosis the result was different and showed an increase levels of Tregs that express CD4+ Foxp3+ T cells but does not express CD25+ T cells (Bonelli et.al.,2009). Bonelli and his colleagues analyzed phenotype and function of CD4+CD25-Foxp3+ T lymphocytes in patients with different autoimmune diseases, they proved that levels of CD4+CD25-Foxp3+ T lymphocytes in patients with SLE was high in comparison with other autoimmune disorders like rheumatoid arthritis(RA) and systemic sclerosis (SS) and healthy control.

Also Huan et. al.,2005 stated that loss of tolerance function is associated with the increase in the incidence of autoimmune disease such as multiple sclerosis(MS) because loss of tolerance mechanism give a chance for pathogenic T cells to proliferate and destruct self cells.

Huan and his colleagues analyzed Foxp3, CD4CD25 expression in patients with MS they reported there was an abnormalities in protein expression of Foxp3+ in CD4+CD25+ T lymphocytes in the blood of MS patients this led to defect in suppression induced during binding of T cell receptor (TCR), as a result impairment in peripheral immune regulation was archieved. So lacking Foxp3 means dysfunction in immune regulation by Tregs and this may lead to MS, this give an insight for therapy , TCR peptide vaccination could be of advantage (Huan et.al.,2005).

In certain cardiac dysfunction the case was different , some researchers(Ammirati et.al., 2010) work on carotid and coronary atherosclerosis they evaluated Treg cells levels using flowcytometry technique by analyzing Foxp3+ and CD4+CD25+ T cells , they found no differences in Tregs levels in patients with acute coronary syndrom,myocardial infarction and chronic stable angina.

From the present result and other researcher results it can be concluded that these markers have no fixed mechanism for many diseases although it has generally with suppressive activities.

Table(3-10): Ranges and means of CD4CD25 levels for each age group

No. of Group	Age Groups	No. of Patients(%)	Male(%)	Female(%)	Ranges of CD4CD25	Mean of CD4CD25
1	2-4	1(2.2)	0	1(2.2)	0.7	0.7
2	8-10	12(26.6)	3(6.6)	9(20)	0.9-33.7	17.98
3	5-7	8(17.77)	3(6.6)	5(11.11)	05-8.3	3.86
4	11-13	20(44.44)	8(17.77)	12(26.6)	1.1-8.8	3.8
5	14-16	2(4.44)	0	2(4.44)	2.9-5.1	4
6	17-19	2(4.44)	1(2.2)	192.2)	1.2-3.4	2.3

Table 3-10 showed means and ranges of CD4CD25 of male and female included in flowcytometry study. Mean values of CD4CD25 of each age group 2-4, 5-7, 8-10, 11-13, 14-16, 17-19 were 0.7, 3.86, 17.98, 3.8, 4, 2.3 respectively, age group 8-10 revealed increased mean value 17.98, in this age group three increased values of CD4CD25 18.3, 27.6 and 33.7 The age group 11-13 had 20 patients (44.44%) of total patients included in the experiment of CD4CD25 in flowcytometry study the highest number of patients. See Figures 3-10, 3-11, 3-12, 3-13, 3-14, 3-15, 3-16 and 3-17show selected results of flowcytometry.

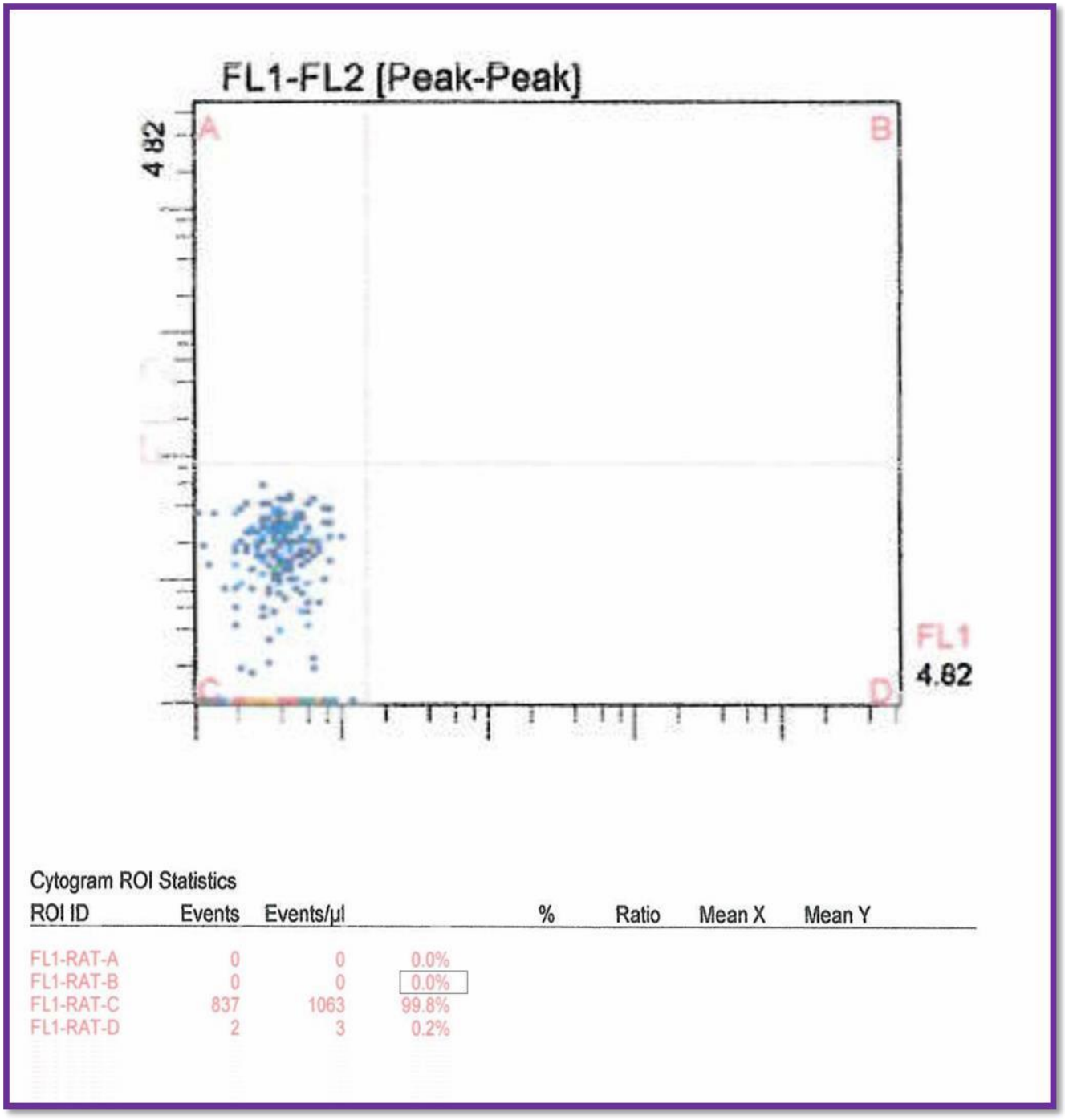
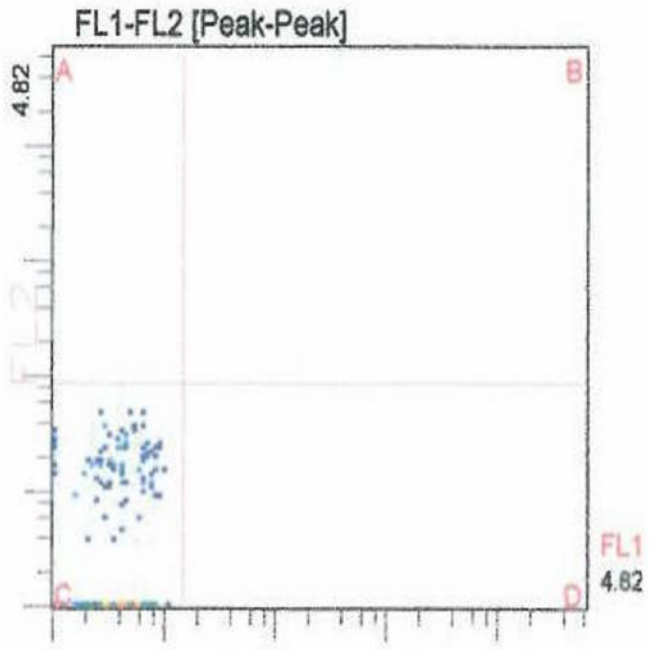


Figure (3-10): Test control of CD4CD25, the value was 0.0%

Table(3-11): Ranges and means of CD4 levels for each age group

No. of Group	Age Groups	No. of Patients(%)	Male(%)	Female(%)	Ranges of CD4	Mean of D4
1	2-4	1(2.2)	0	1(2.2)	1.7	1.7
2	5-7	8(17.77)	3(6.6)	5(11.11)	0.5-2.9	1.95
3	8-10	12(26.6)	3(6.6)	9(20)	1.9-23	12.11
4	11-13	20(44.44)	8(17.77)	12(26.6)	2.5-22	12.43
5	14-16	2(4.44)	0	2(4.44)	0.9-1.5	1.65
6	17-19	2(4.44)	1(2.2)	192.2)	0.2-7.3	3.85

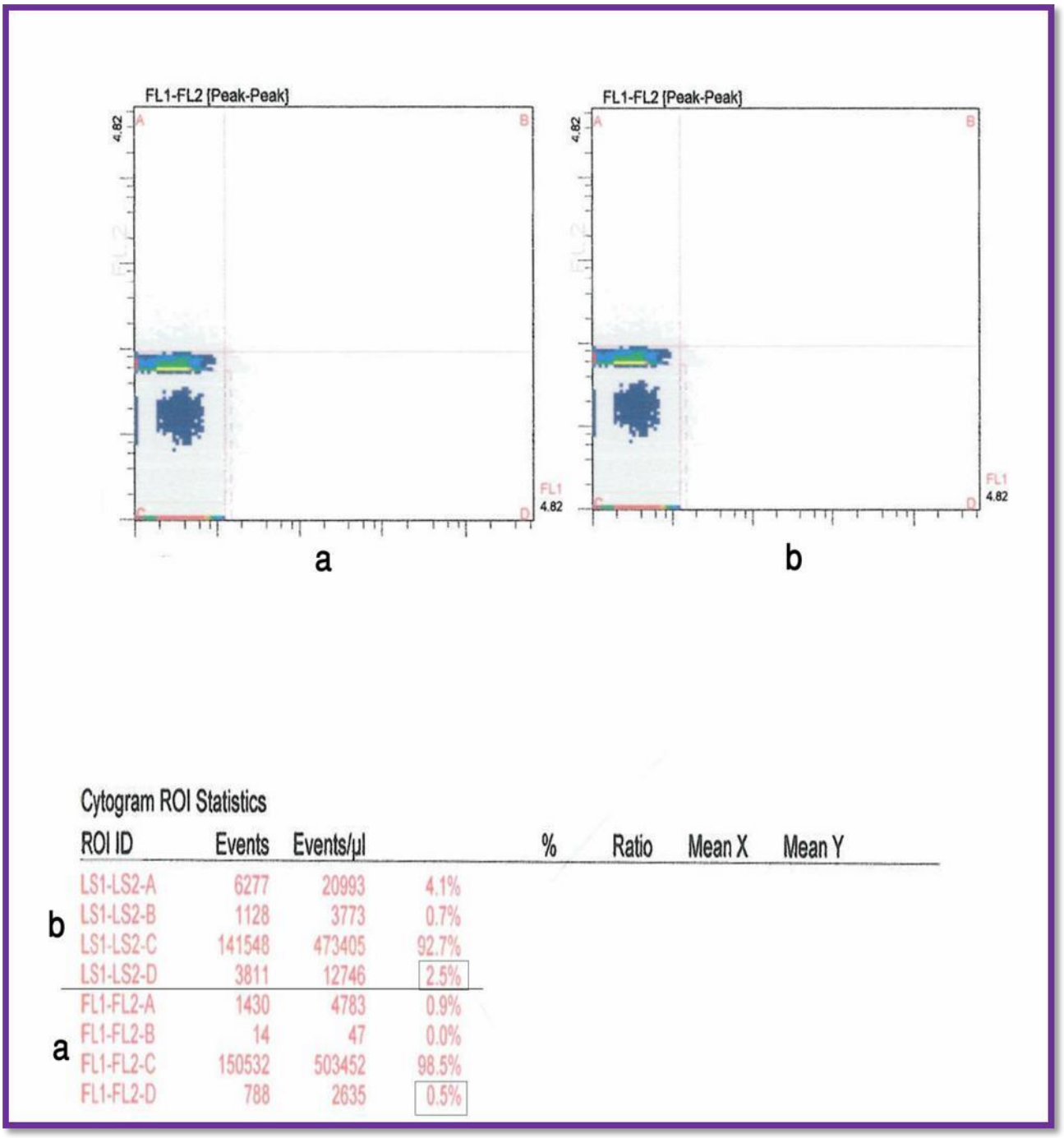
Table 3-11 showed means and ranges of CD4 of male and female included in flowcytometry study. Mean values of CD4 of each age group 2-4, 5-7, 8-10, 11-13, 14-16, 17-19 were 1.7, 1.95, 12.11, 12.43, 1.65, 3.85 respectively. Age group 8-10 and 11-13 revealed increased mean value 12.11 and 12.43 respectively, in the age group 8-10 three increased values of CD4 23,22 and 17.5 also in the age group 11-13 there are two increased levels both with 22 value. The Age group 11-13 had 20 patients (44.44%) of total patients included in the experiment of CD4 in flowcytometry study, represents the highest number of patients with the highest mean of CD4. No available data represented this results, and it is suggested to do further investigations to confirm and explain this results. See figures 3-18, 3-19, 3-20 and 3-21of flowcytometry results to compare CD4 levels in different test groups compared to control.



Cytogram ROI Statistics

ROI ID	Events	Events/µl	%	Ratio	Mean X	Mean Y
FL1-FL2-A	1	1	0.2%			
FL1-FL2-B	0	0	0.4%			
FL1-FL2-C	565	739	99.5%			
FL1-FL2-D	2	3	0.0%			

Figure(3-18): Test control of CD4, the value was 0.0%



Cytogram ROI Statistics

	ROI ID	Events	Events/µl	%	Ratio	Mean X	Mean Y
b	LS1-LS2-A	6277	20993	4.1%			
	LS1-LS2-B	1128	3773	0.7%			
	LS1-LS2-C	141548	473405	92.7%			
	LS1-LS2-D	3811	12746	2.5%			
a	FL1-FL2-A	1430	4783	0.9%			
	FL1-FL2-B	14	47	0.0%			
	FL1-FL2-C	150532	503452	98.5%			
	FL1-FL2-D	788	2635	0.5%			

Figure (3-20): a. The lower level of CD4 of group number 4 the value was 0.5%
b. The lower level of CD4 of group number 2, the value was 2.5%

Table(3-12): Ranges and means of CD25 levels for each age group

No. of Group	Age Groups	No. of Patients(%)	Male(%)	Female(%)	Ranges of CD25	Means of CD25
1	2-4	1(2.2)	0	1(2.2)	1	1
2	5-7	8(17.77)	3(6.6)	5(11.11)	1.2-8.3	5.3
3	8-10	12(26.6)	3(6.6)	9(20)	3.4-28.9	16.45
4	1-13	20(44.44)	8(17.77)	12(26.6)	1.2-16.8	9.93
5	14-16	2(4.44)	0	2(4.44)	1.1-3.3	2.3
6	17-19	2(4.44)	1(2.2)	192.2)	2-3.6	2.8

In table 3-12 mean values of CD25 of each age group 2-4, 5-7, 8-10, 11-13, 14-16, 17-19 were 1, 5.3, 16.45, 9.93, 2.3 and 2.8 respectively, age group 8-10 revealed increased mean value of 16.45, in the age group 8-10 there were 4 increased values of CD25: 28.9,27,14.1 and 13.3.(Figures 3-22, 3-23, 3-24, 3-25, 3-26, 3-27 and 3-28).

The explanation for the increased levels in Treg cells may due to the reason stated in Filippi and von Herrath in 2007 whom they said that although the mechanism by which viruses might induce autoimmunity is not understood, viral infections might be capable of "unmasking" β-cells for recognition by CD8+ T-cells by promoting interferon production and upregulation of major histocompatibility complex (MHC) class I molecules on β-cells. These events combined may be sufficient to condition the pancreatic islets for autoimmune attack, and they proposed that not only viral infections cause the initiation autoimmunity but rather act to provide a "fertile field" for further expansion of activated autoreactive T-cells, leading to autoimmune disease (Filippi and von Herrath , 2007).

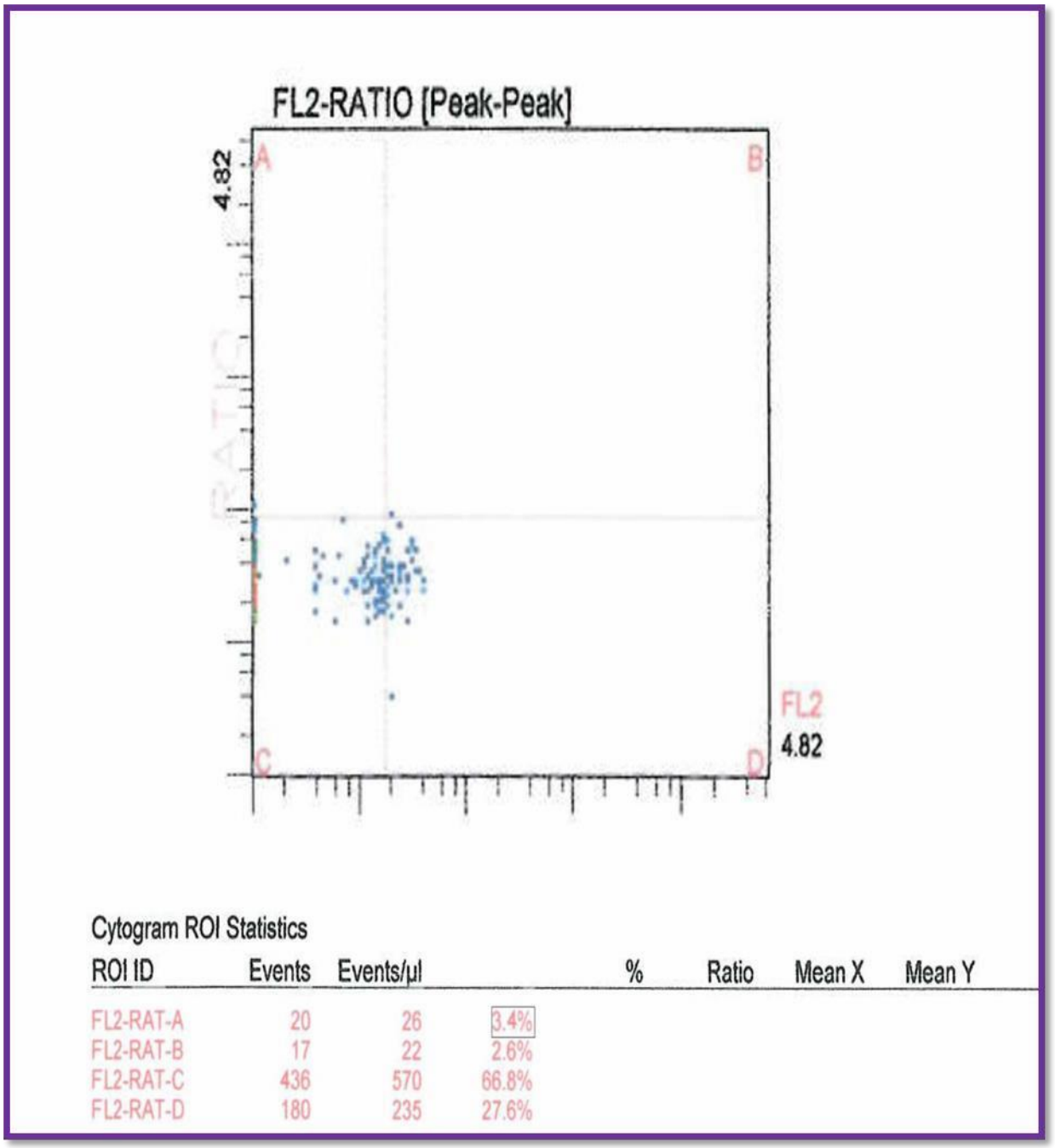
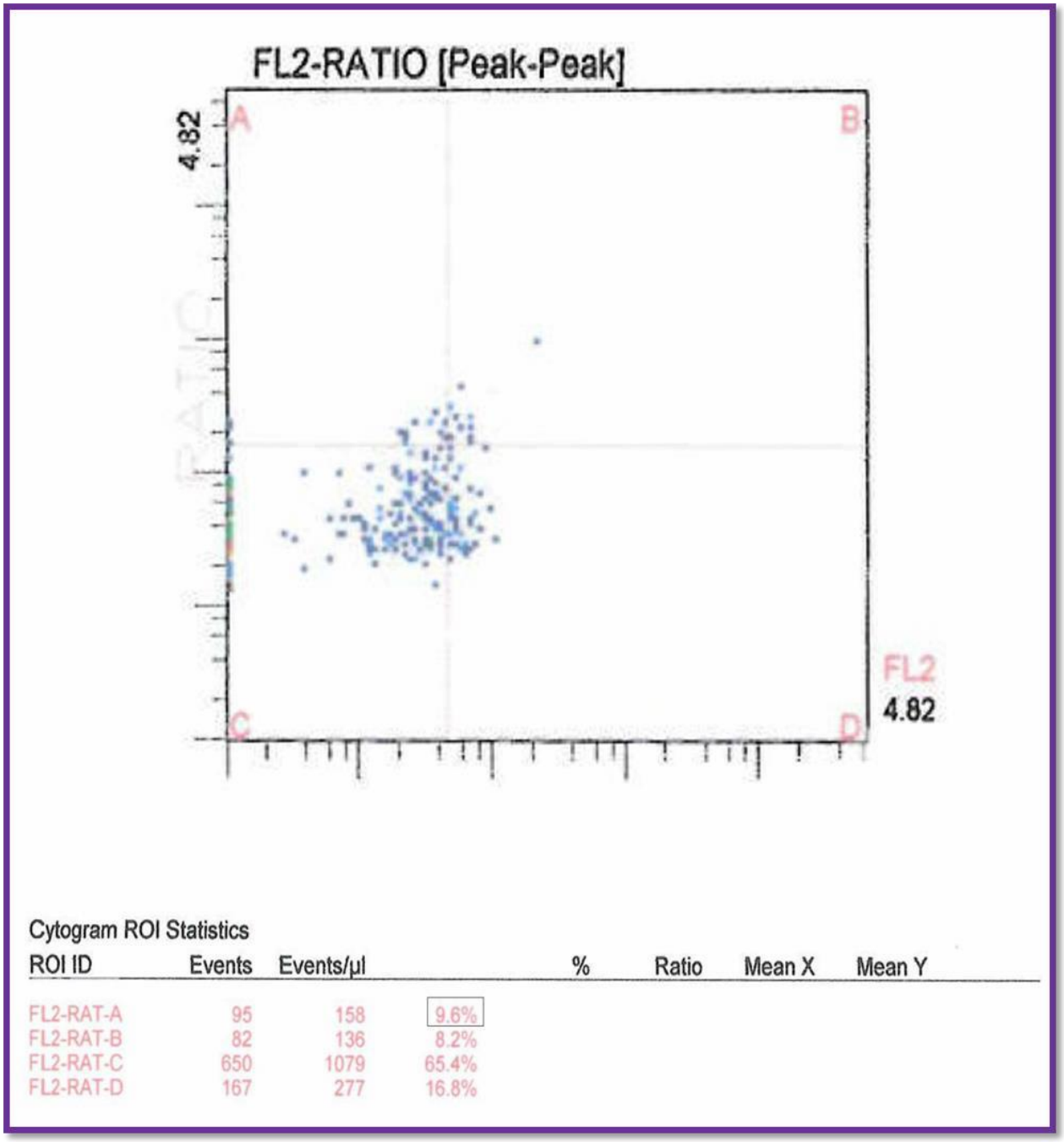


Figure (3-23): The lower level of CD25 of group number 3, the value was 3.4

Figure (3-24): The approximate mean of CD25 of group number 4, the value was 9.6%



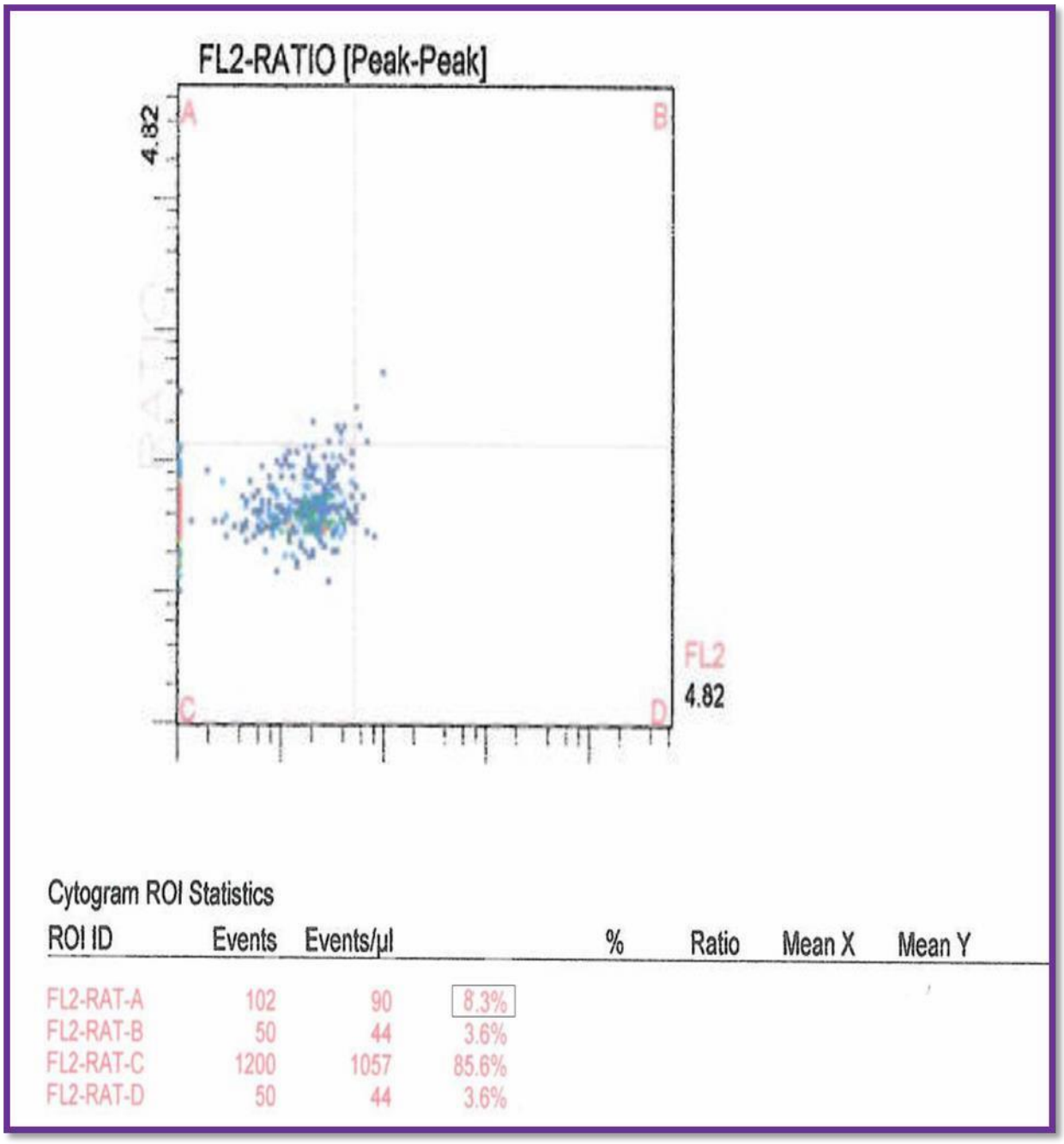


Figure (3-25): The approximate mean of CD25, the value was 8.3%

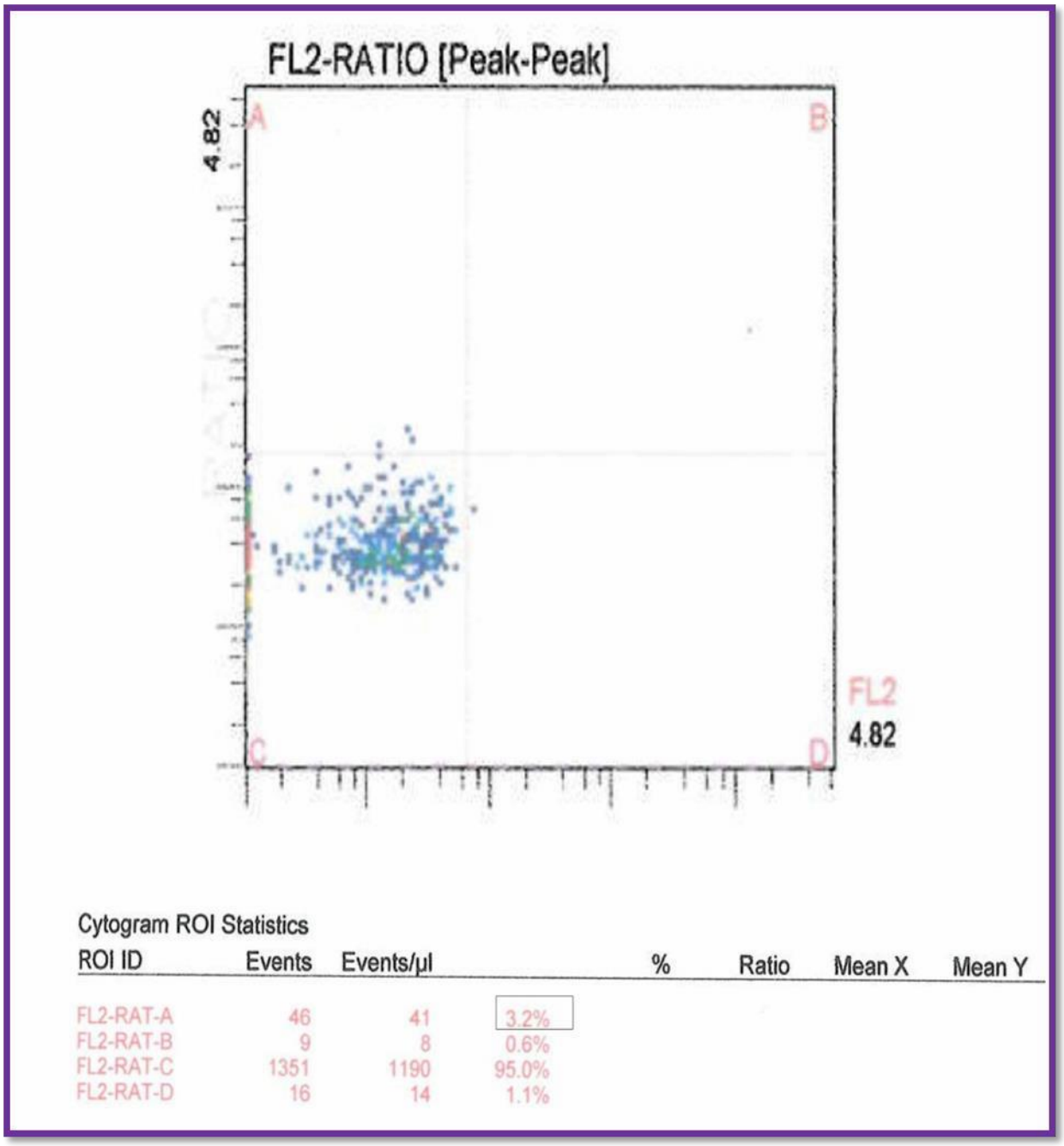


Figure (3-26): Flowcytometry results of CD25 of selected case of T1D patient who had the higher level in group number 5, the value was 3.2%

Luczyriski et.al., 2009 stated that no disturbances in the percentage of Tregs in patients with T1D due to diminished expression of some elements important in Treg function. Numerous studies have demonstrated the key role of CD4+CD25+Foxp3+ regulatory T cells the development of T1D. However the changes in Tregs expression and function in addition to the regulation of these processes until that time not clearly understood. The present project studied the percentage of Tregs in the peripheral samples. Peripheral studies for Tregs in T1D are mostly used rather than tissue based studies because of the difficulties in obtaining tissues and histological data to support the action of different types of Tregs in T1D(Tan et.al.,2014). From the above reference results it can be

concluded that functions of Tregs is more significant in the development of T1D rather than their frequencies. Also Luczyriski et.al.,2009 demonstrated that there is a disturbances in the function of Tregs rather than the frequency of these cells. The increase in the percentages of Tregs in this study (tables 3-6,3-7 and 3-8) might be a reaction of the host immune system to compensate the down regulation and diminished expression of some elements important in Treg function against autoimmune reaction or viral infection. Kukreja et.al.,2002 found that the function of Tregs in the onset of T1D is suboptimal and conclude that there were a multiple defects in immunoregulatory mechanism in T1D suggesting that there are cells other than Tregs might have a role in immuneoregulatory mechanism. According to this reference choosing the time of the study is considered as a detrimental factor for the type of results obtained which definitely change at a later time in the same patient.

Regulatory T cells come in many forms with the most common that express CD4CD25 and Foxp3 (CD4+CD25+ regulatory T cells) which they are differ from T helper cells (Hori et.al.,2003). Foxp3 can be used as a good marker for mouse CD4+CD25+ T cells, although recent studies have also shown evidence for Foxp3 expression in CD4+CD25- T cells. In addition, Foxp3 is also expressed by recently activated conventional T cells and thus does not specifically identify human T-reg (Sakaguchi et.al.,2004).

Numerous studies have demonstrated the key role of CD4+CD25+Foxp3+ regulatory T cells the development of T1D. However the changes in Tregs expression and function in addition to the regulation of these processes until that time not resolved(Tan et.al.,2014). From the above reference, it can be concluded that functions of Treg is more significant in the development of T1D rather than their frequencie The chief function of Treg cells in a normal person is to maintain immune homeostasis in the lymphoid organs. After the onset of immune response. Tregs cells use additional suppressive stratigies to resolve inflammation and limit tissue damage. TGF-B produced by Tregs cells "feed back" to stabilize and hence Foxp3 expression in Treg cells there by boosting their suppression function. As it is shown in Table 3-6 there is a significant differences between control and all parameters studied these results are in agreements with Pihl et.al. in 2011who stated that the increased expression of regulatory T cells-associated markers are obtained from individuals with early development of autoimmunity. While Lindely et.al in 2005 and Brusko et.al in 2007 couldn't find any change in the values of CD4+CD25+ T cells between healthy and diabetic persons. On other hand the results of the present study differs from Zahran et.al. in 2012 who mentioned that levels of Foxp3+ CD4+CD25+ T cells were decreased in children with T1D, the difference between Zahran study and the present study comes from that the samples in the recent study were collected from newly diagnosed individuals and the increase in the Tregs may be due to chronicity of the disease .

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