

## RESEARCH ARTICLE

### Biochemical Alteration and Immunohistochemical Characterization of Lymphoid Cells Involved in Type 2 Diabetes Mellitus

Nahla H. El-shaer

Zoology Department, Faculty of Science, Zagazig University, Zagazig, Egypt

#### ABSTRACT

T cells are crucial for the inflammation development of metabolic disorder and insulin resistance and the cytokines of T cells are considered as an excellent evaluating tools for the immune system activity in many diseases. As cytokine profile associates with the development of type-2 diabetes mellitus (T2-DM) is well understood the link between this disease and the involved immune cells still unclear. Therefore, it was hypothesized that Th1 pathway is disrupted in the pathogenesis of type 2DM. As such, we aimed to evaluate IL-12, which induces Th1 pathway, and IL-2 which mediates Th1 function. Paraffin-embedded sections of peripheral blood lymphocytes with T2-DM patients were stained with antibodies against CD4 and CD8. Computerized image analysis was used to calculate areas of stained lymphocytes taken from diabetic and control patients. Clinical data and blood samples were collected from Eighty-one (37.6±13.9 years old) diabetic Egyptian patients and 103 (40.5±12.7 years old) of normal control volunteer to evaluate the level of serum interleukin IL-2 and IL-12 in their blood by using ELISA technique. Routine laboratory analysis, including fasting blood glucose, CRP, lipid profile, fasting insulin, and CBC for each patient were measured. Immunohistochemical results in 2DM showed an increase in the numbers of CD8<sup>+</sup> cells with a decrease in the numbers of CD4<sup>+</sup> cells displayed. Diabetic patients showed decreased and increased values of IL-2 and IL\_12, respectively as compared to control values. Conclusion: This data indicate the importance of these two cytokines in the pathogenesis of type-2 Diabetes mellitus.

**Keywords:** T2-DM, CD8<sup>+</sup>, CD4<sup>+</sup>, immunohistochemical.

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#### Correspondence to:

Dr. Nahla H.El-shaer

Lecturer of Histology and Histochemistry

Zoology Department, Faculty of Science, Zagazig University, Egypt

Tel: +201006140559

E-Mail:

nahlaelshaer@yahoo.com

#### INTRODUCTION

Type 2 diabetes (2DM) causes disturbance of the immune system and is characterized by impaired insulin secretion resulting in serious complications. There is a rapid rise in both social and economic problems with a terrible impact on the global situation, where more than a third of the world's population is diabetic; most of them are unaware of initial symptoms of the disease (Wild *et al.*, 2004). It is accompanied by short and long-term complications on all arteries, and thus harmful to all body organs like, associating with

nephron- and retinopathy which are manifested by inflammatory process and the probability to frequent infection (Fowler, 2011), so that inflammation is considered as a major driving force in T2-DM and associated complications (Guarner and Rubio-Ruiz, 2014).

2DM is an autoimmune disease which is characterized by destruction of insulin-producing pancreatic  $\beta$ -cells located in the pancreatic islets of Langerhans (Tisch and McDevitt, 1996). This destruction requires both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets (Nagata *et*

*al.*, 1994). Other studies also suggested an important role for CD8<sup>+</sup> T cell subset in the early induction phase of ID-DM (Wong *et al.*, 1996). This may be attributed to  $\beta$ -cells, which are destroyed by cytotoxic CD8<sup>+</sup> T cells and lead to the release of pancreatic antigens that lead to a subsequent activation of CD4<sup>+</sup> T cells. Although CD4<sup>+</sup> T cells may not damage class II-negative  $\beta$ -cells directly (Serreze and Leiter, 1994), they can recruit other activated macrophages, that can damage  $\beta$ -cells by secreting IL-1 $\alpha$  and possibly other cytokines (Von Herrath and Oldstone, 1997). In addition, CD41 T cells can expand or recruit other CD81 T cells. Thus, cytotoxicity by CD81 T cells is believed to be potential to the onset of diabetes.

CD4<sup>+</sup> helper cells and CD8<sup>+</sup> cytotoxic cells are 2 phenotypes of T lymphocytes, each have a distinct surface markers found in lymph nodes and also circulate in the blood. A wide heterogeneity exists in these cells because of sex, age, ethnicity, genetics, exposures, and infections (Wikby *et al.*, 2008). Mature CD4<sup>+</sup> thymocytes, CD4<sup>-</sup> and CD8<sup>-</sup> of double negative thymocytes are capable of inhibiting the transfer of diabetes. This thymocyte population includes natural killer (NK) T cells, and the role of NK T spleen cells in the regulation of diabetes development is declared (Hammond *et al.*, 1998).

The normal CD4/CD8 ratio can be inverted through apoptotic or targeted cell death of circulating CD4 cells, resulting in expansion of CD8 cells. In addition, the low CD4/CD8 ratio and an immune risk phenotype, is associated with altered immune function, immune senescence, and chronic inflammation (Serrano-Villar *et al.*, 2014 and Serrano-Villar *et al.*, 2014) and may predict morbidity and mortality (Hupper *et al.*, 2003). Besides, the abnormal ratio of CD4/CD8 is not uniformly present in all autoimmune diseases (Carvajal *et al.*, 2017). A low CD4/CD8 ratio can reflect  $\beta$ -cell destruction and used to predict diabetes diagnoses associated with a wide-range of pathology (Al-Sakkaf *et al.*, 1989). Using nasal administration with a combination of GAD65, peptides was found to alter Th1/Th2 cell

balance in insulin-dependent diabetes (Tian *et al.*, 1996).

IL-2 is a Th1 pro-inflammatory cytokine. It has a great role in T cells activation and thus production of interferon gamma (IFN- $\gamma$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ), which improves the activity of NK (Lenardo, 1991 and Sakaguchi *et al.*, 2008). Accordingly, several studies have used IL-2 as a therapy to motivate the immune system and its responses (Smith and Humphries, 2009). Cytokines play a role in the pathogenicity of many disorders including metabolic, infectious, autoimmune and inflammatory syndromes (Lunney *et al.*, 1998). Thus, IL-2 participates in immune functions and its work contributed to the creation and proliferation of antigen-specific immune responses (Raeburn *et al.*, 2002).

IL-12 is a pro-inflammatory cytokine. It consists of mainly two subunits p35 and p40 produced by antigen presenting cells in response to pathogen-associated molecular patterns (PAMPs) and to danger-associated molecular patterns (DAMPs). It makes stimulations that switch off the immune response towards Th1 reactions, and thus makes protection against endogenous microbes (Watford *et al.*, 2003 and Hölischer, 2004).

There is no any previous reviews elucidate the immunopathology of diabetes and evaluate the accurate role of IL-2 and IL-12 cytokines in the risk of type-2 diabetogenesis. Therefore, the aim of the present work is focused to obtain a deeper understanding of peripheral blood lymphocytes in diabetes immunopathology by using immunohistochemical staining for CD4, CD8 (all on T lymphocytes). In addition, the role and relationship of interleukins-2 and 12, the two important cytokines two opposing immune functions were investigated in this study of in type-2 diabetogenesis patients.

## SUBJECTS AND METHODS

### Subjects

This work was done at Zagaig University, Egypt. The patients included in this study were classified into two main groups. The first group included 81 patients with type 2 *Diabetes mellitus* and were chosen from the outpatient

endocrinology clinic, Zagazig University Hospitals during the period from April 2015 to May 2017. The second control group included 103 volunteers with matched age and sex who were healthy and without history of diabetes in their families. All subjects with DM and healthy volunteers were subjected to full history, clinical examination, and routine investigations for complete blood count (CBC). In addition, written consents were taken from all volunteer participants in the study.

Type-2 DM was diagnosed in accordance to WHO guidelines, 1998 (Alberti and Zimmet, 1998). In addition, the Ethics Committee of Zagazig University according to the 1964 declaration of Helsinki (World Medical Association Declaration of Helsinki, 2014), approved this study. Patients did not suffer from chronic inflammation, cancer, allergy and autoimmune diseases.

#### Laboratory analysis

Fasting 8h blood samples were withdrawn from both diabetic and healthy subjects to evaluate concentrations of fasting glucose and other 12 h samples for lipid profile investigation. Fasting glucose was measured using the glucose oxidase method, and the fasting serum lipid profile (cholesterol, triglyceride, HDL) was done by enzymatic colorimetric technique. Serum LDL was calculated using the formula of (Jialal *et al.*, 2017).

#### Assessment of blood glucose level and insulin

(A) Fasting insulin, fasting blood glucose (FBG), and glycated hemoglobin (HbA1c) were measured by using collected whole blood. (B) FBG was detected by using device (COBAS INTEGRA® 400 plus, Roche Diagnostics Ltd, Switzerland). HbA1c level was detected using Siemens DCA analyzer. (D) Fasting insulin level was also detected with a human insulin antibody clone using ELISA kit (EMD Millipore, Billerica, USA). Fasting insulin and fasting glucose levels were used to calculate both insulin resistant state and  $\beta$ -cell function (Matthews *et al.*, 1985). Assessment of insulin resistance (HOMA-IR):  $\text{HOMA-IR} = [\text{Fasting insulin } (\mu\text{IU/mL}) \times \text{Fasting glucose (mmol/L)}] /$

22.5. Homeostatic model assessment of  $\beta$ -cell function (HOMA- $\beta$ ):  $\text{HOMA-}\beta = [20 \times \text{Fasting insulin } (\mu\text{IU/mL})] / [\text{Fasting glucose (mmol/L)} - 3.5]$ .

#### Assessment of lipid profiles

The colorimetric enzymatic method was done using a device (COBAS INTEGRA® 400 plus, Roche Diagnostics Ltd, Switzerland) to detect triglyceride (TG), total cholesterol (TC), and high density lipoprotein cholesterol (HDL-c) levels. Low density lipoprotein cholesterol (LDL-c) level was calculated using the Friedewald equation as follows:  $\text{LDL-c} = \text{total cholesterol} - (\text{HDL-c} + \text{TG} / 5)$  (Jialal *et al.*, 2017).

#### Complete blood count analysis

Whole blood was taken from the donor into EDTA tubes. The count of white blood cells (WBCs), red blood cells (RBCs), and platelets with their indices were determined by an autoanalyzer (sysmes XS-500i, Germany).

#### Detection of serum CRP

Serum C-reactive protein (CRP) was detected using an immunoturbidimetric method, which applied on an analyzer (COBAS INTEGRA® 400 plus, Roche Diagnostics Ltd, Switzerland). The detected sensitivity limit of this assay is 0.07 mg/L.

#### Detection of Serum IL-2 and IL-12

All blood sera samples were collected and also stored at  $-4^{\circ}\text{C}$  for the measurement of both interleukins (IL-2 and IL-12) using enzyme-linked immunosorbent assay (ELISA) technique (AviBion Human IL-2 and IL-12 EASIA kits). Samples were then brought to room temperature and processed according to the manufacturer's instructions. The color of the reaction mixture was detected at 450 nm. Finally, a standard curve was plotted between concentrations used and the corresponding absorbance, and then the results were calculated using Excel.

#### Immunohistochemistry analysis

Paraffine blocks of peripheral blood were prepared according to (Thapar *et al.*, 2009).

The blood tissue was fixed in a 1:1 solution of 10% alcohol: formalin for one hour. Fill the tissue in a centrifuge tube and spin down at 2500 rpm for 10–15 minutes. The supernatant was poured off and the sediment was drained off on a filter paper. The sediment is routinely processed by the paraffin embedding technique Processes of dehydration with four changes of ethanol, clearing with 1:1 ethanol/xylene (1 hour); then with xylene in three changes (1 hour/each), infiltration and embedding by paraffin wax were performed. Section cutting was performed by usual method at 3  $\mu$ m in thickness, mounted and prepared for further histochemical examination (Suvarna *et al.*, 2018).

#### Immunohistochemical technique

The standard immunohistochemical methods were adopted in routine procedures (Eissa and Shoman, 1998). The tissue sections were routinely microwave-treated to un-mistake the epitopes of antigen (Cattoretti *et al.*, 1992) The demonstration of antigen in tissues by immune-staining is a two-step process. The first step is binding of the primary monoclonal anti-CD<sup>4</sup>, diluted 1:2 (Ventana/Nova Castra, Tucson, AZ), monoclonal anti-CD<sup>8</sup> (clone C<sup>8</sup>/144B) diluted 1:10 (DAKO-Cytomation, Carpinteria, CA) to the related antigen, followed by visualization of this reaction by a secondary or link antibody to which are attached different enzyme systems, which are collectively known as the universal. The primary antibody determines the specificity of the reaction; whereas, the secondary antibody, with its linked enzyme, causes amplification of the reaction, hence, increase of the sensitivity of the test. The Biotin-Streptavidin (BSA) system was used to visualize the markers (32). Diaminobenzidine (DAB) was used as chromogen since it allows a permanent preparation. Hematoxylin counterstain was done using Mayer's hematoxylin (Hx). the specimens were mounted using mounting medium Canada balsam and examined by the microscope (Olympus DP 71).

#### Statistical analysis

All statistical analyses were done by using MedCalc 15.8, GraphPad Prism 5.0 and SPSS 17.0. All data were reported as means  $\pm$  SD. Differences between groups were tested using Kruskal-Wallis and Mann-Whitney U tests (for continuous variables and nonparametric analyses for independent samples, respectively). Correlation coefficients (r) was done by using nonparametric Spearman's correlation analysis odds ratios and 95% confidence intervals (CI) was calculated to find the association between inflammatory markers and T2-DM. However, the P value < 0.05 was considered statistically significant.

#### RESULTS

Clinical investigation of subjects enrolled in this study revealed no significant differences in age and male/female ratio between the patients and controls (P < 0.05). The mean duration of illness for the patients group was 11.5 $\pm$ 4.8 years. Neither patients nor controls had a history of clinical findings of impaired hepatic or renal function or any parasitic infection. Several clinical, biochemical and inflammatory parameters were measured for both patients and control. There was a significant difference between patients and controls in triglyceride level, white blood cells count, QUICKI and CRP level. Moreover, there was a high significant difference between patients and controls in the level of Homa-IR and Homa- $\beta$ . There was a high significant difference between patients and controls in the level of Hba1c, fasting glucose and insulin levels.

Regarding IL-2 and IL-12 levels, differences in means were observed in both IL-2 level (170.17  $\pm$  52.47 for patients and 207.7  $\pm$  76.32 for controls) and IL-12 (147.11  $\pm$  63.66 for patients and 67.38  $\pm$  26.55 for controls). Our results showed that the patients have lower IL-2 levels and higher IL-12 levels than control but with no significant value (Table 1).

Table 1: Clinical, biochemical, and inflammatory parameters in both diabetic and control subjects.

| Parameters                              | Patients (n= 81) | control (n=103) | P value   |
|---|------------------|-----------------|-----------|
| Age (years)                             | 37.6±13.9        | 40.5±12.7       | NS        |
| Gender (m/f)                            | 54/27            | NS              | NS        |
| Body mass index (kg/m <sup>2</sup> )    | 26.8±6.6         | 25.7±4.5        | NS        |
| Systolic blood pressure (mmHg)          | 125.70±17.91     | 114.40±15.67    | NS        |
| Diastolic blood Pressure (mmHg)         | 75.31±13.58      | 74.41±9.79      | NS        |
| Duration of disease (years)             | 11.5±4.8         | --              | --        |
| Cholesterol (mg/dl)                     | 201±45.81        | 186.78±33.87    | NS        |
| HDL cholesterol (mg/dl)                 | 43.37±9.16       | 57.63±8.42      | NS        |
| LDL cholesterol (mg/dl)                 | 137.30±17.47     | 129.78±12.56    | NS        |
| Triglycerides (mg/dl)                   | 193±38.52        | 132.51±43.12    | <0.05*    |
| Hemoglobin (g/dl)                       | 14.56±3.65       | 13.56±3.22      | NS        |
| White blood cells (10 <sup>3</sup> /cm) | 10.3 ± 2.96      | 6.3 ± 2.1       | NS        |
| Red blood cells (10 <sup>6</sup> /cm)   | 5.6±1.22         | 5.8±1.8         | NS        |
| Platelets (10 <sup>3</sup> /cm)         | 325±67.98        | 370±80.7        | NS        |
| Hba1C (%)                               | 9.9±2.5          | 5.93±0.8        | <0.001*** |
| Fasting glucose (mg/dl)                 | 229.83 ± 70.93   | 99.4 ± 11.097   | <0.001*** |
| Fasting insulin (μU/ml)                 | 29.43±8.00       | 8.97±2.98       | <0.001*** |
| Homa-IR                                 | 5.80±1.584       | 2.82±0.77       | <0.01**   |
| Homa-β                                  | 346.85±187.53    | 172.54±68.72    | <0.01**   |
| QUICKI                                  | 0.27±0.03        | 0.38±0.01       | <0.05*    |
| CRP (mg/L)                              | 10.8±0.2         | 4.2±0.13        | <0.05*    |
| IL-2 (pg/ml)                            | 170.17 ± 51.47   | 207.7 ± 75.32   | NS        |
| IL-12 (pg/ml)                           | 147.11 ± 62.66   | 67.38 ± 25.55   | NS        |

All data are represented as mean ± SD. \*  $P < 0.05$  is significant, \*\* $P < 0.01$  is high significant, \*\*\* $P < 0.001$  is very high significant and NS is non-significant value. HDL = high-density lipoprotein, LDL = low-density lipoprotein, TG= triglyceride, CRP = C- reactive protein, IL-2= Interleukin-2, IL-12= Interleukin-12. Hba1c = glycohemoglobin, HOMA-IR (Homeostasis Model Assessment - Insulin Resistance) = [fasting insulin ((μU/ml) x fasting glucose (mg/dl))/405. HOMA-β (Homeostasis Model Assessment-β) = (fasting insulin (μU/ml) x 360) / (fasting glucose (mg/dl) - 63), QUICKI (Quantitative insulin sensitivity check index) = 1/ [log (fasting insulin (μU/ml)) + log (fasting glucose (mg/dl))]

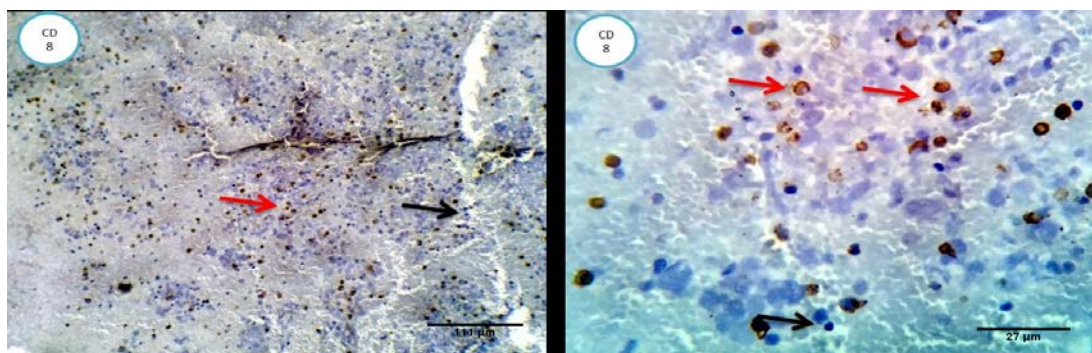


Figure 1. Two Photomicrographs of Cell block tissue sections showing about 80-85% positive lymphoid cells for CD8. Scale bars correspond to 111 μm & 27 μm, respectively.

Table 2: Comparison among diabetic patients as regards the duration of illness, age, and sex, body mass index, control of diabetes and other related indices.

| Parameters      | DM duration                                 |   |                                    | p      |
|-----------------|---|---|------------------------------------|--------|
|                 | 5-10 years (n=40)                           | 10-20 years (n=27)                        | > 20 years (n= 14)                 |        |
| Glucose (mg/dl) | 162.5±54.8                                  | 196.8±76.9                                | 241.6±40.9                         | 0.045* |
| Hba1c %         | 7.1±3.6                                     | 8.2± 3.8                                  | 9.6±4.67                           | 0.034* |
| IL-2 (pg/ml)    | 67.9±30.7                                   | 256.8±105.8                               | 407.9± 187.9                       | 0.034* |
| IL-12 (pg/ml)   | 36.7±14.5                                   | 91.7± 34.5                                | 169.9± 56.8                        | 0.039* |
|                 | Age   |   |                                    | P      |
|                 | 15-35 years (n= 37)                         | 35-50 years (n=20)                        | > 50 years (n= 34)                 |        |
| Glucose (mg/dl) | 193.9± 54.9                                 | 190.9±67.9                                | 236.8± 70.7                        | 0.064  |
| Hba1c %         | 8.34± 2.5                                   | 8.99± 3.5                                 | 10.45± 4.1                         | 0.074  |
| IL-2 (pg/ml)    | 100.8±45.7                                  | 146.8±45.8                                | 190.9±67.9                         | 0.32   |
| IL-12 (pg/ml)   | 69.9±38.7                                   | 98.9±31.3                                 | 189.9± 76.2                        | 0.073  |
|                 | Sex   |   | p                                  |        |
|                 | Males (n= 54)                               | Females (n= 27)                           |                                    |        |
| Glucose (mg/dl) | 199.7± 67.8                                 | 192.5± 80.6                               | 0.864                              |        |
| Hba1c %         | 8.9± 4.2                                    | 9.4±3.1                                   | 0.667                              |        |
| IL-2 (pg/ml)    | 146.6±67.9                                  | 164.9±68.9                                | 0.99                               |        |
| IL-12 (pg/ml)   | 157.7±78.9                                  | 149.9±88.9                                | 0.84                               |        |
|                 | Body mass index                             |   |                                    | p      |
|                 | Normal weight < 25 g/m <sup>2</sup> (n= 30) | Overweight 25-30 kg/m <sup>2</sup> (n=45) | Obese > 30 kg/m <sup>2</sup> (n=6) |        |
| Glucose (mg/dl) | 188.7± 57.8                                 | 191.5± 80.6                               | 206.9±90.8                         | 0.325  |
| Hba1c %         | 8.1± 3.2                                    | 8.7±3.7                                   | 8.6± 4.8                           | 0.146  |
| IL-2 (pg/ml)    | 148.7±57.9                                  | 183.9±69.7                                | 203.9±79.1                         | 0.179  |
| IL-12 (pg/ml)   | 146.8±88.2                                  | 168.9±89.2                                | 199.9±89.2                         | 0.097  |
|                 | Control of diabetes                         |   |                                    | p      |
|                 | Good control Hba1c 6-7.9% (n = 23)          | Fair control Hba1c 8-9.9% (n = 40)        | Poor control Hba1c ≥10% (n =18)    |        |
| Glucose (mg/dl) | 149.9± 34.9                                 | 188.8± 54.9                               | 238.8± 78.9                        | 0.013* |
| IL-2 (pg/ml)    | 108.8±55.7                                  | 166.8±48.8                                | 207.9±67.9                         | 0.064  |
| IL-12 (pg/ml)   | 87.9±78.7                                   | 108.9±71.3                                | 287.9± 76.2                        | 0.062  |
|                 | Fasting glucose                             |   |                                    | p      |
|                 | 101-200 mg/dl (n= 62)                       | 201-300 mg/dl (n=12)                      | > 300 mg/dl (n= 7)                 |        |
| Hba1C (%)       | 8.5 ± 3.7                                   | 7.9± 3.7                                  | 11.6± 4.8                          | 0.037* |
| IL-2 (pg/ml)    | 103.6±51.7                                  | 176.8±38.8                                | 230.9±57.9                         | 0.065  |
| IL-12 (pg/ml)   | 109.9±78.7                                  | 158.9±71.3                                | 298.9± 86.2                        | 0.079  |
|                 | Homa-IR                                     |   |                                    | p      |
|                 | 2-3 (n= 53)                                 | 3.1-5 (n= 20)                             | > 5 (n= 8)                         |        |
| Fasting glucose | 166.8± 43.6                                 | 200.8± 23.7                               | 256.8± 78.9                        | 0.033* |
| Hba1C (%)       | 8.75± 4.3                                   | 9.7± 4.6                                  | 11.1± 4.9                          | 0.049* |
| IL-2 (pg/ml)    | 91.6±41.7                                   | 186.8±34.2                                | 288.9±56.2                         | 0.06   |
| IL-12 (pg/ml)   | 104.9±61.7                                  | 188.9±51.2                                | 278.9± 79.2                        | 0.076  |

All data are represented as means ± SD, \* P < 0.05 is significant, IL-2 = Interleukin-2, IL-12 = Interleukin-12. Hba1c = glycohemoglobin, Homa-IR = hemostasis model of assessment for insulin resistance.

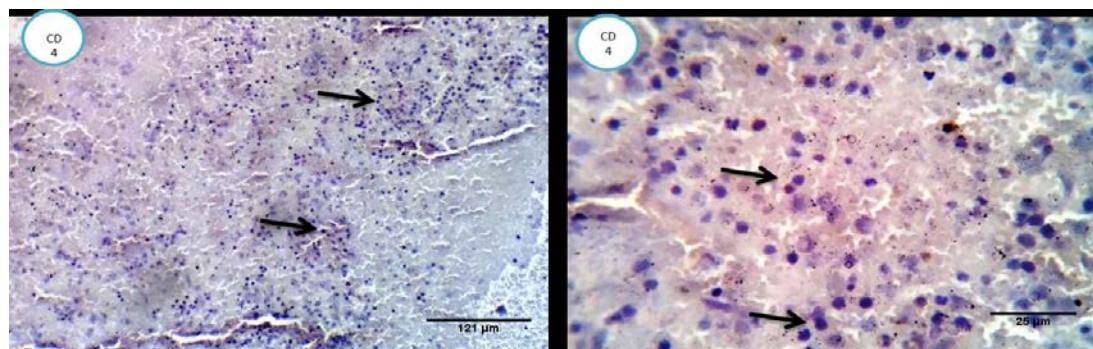


Figure 2. Two Photomicrographs of Cell block tissue sections showing about 90-95% negative lymphoid cells for CD4. Scale bars correspond to 121 µm & 25 µm, respectively.

**Comparative analysis by using independent factors**

A comparison between diabetic patients in this study was done according to different independent factors such as (age, duration of disease, body mass index , gender, diabetes control, Homa-IR, and fasting glucose) , and it was found that type 2 diabetes patients can be affected only by duration of the disease. There were significant differences in the fasting glucose level, Hba1c, IL-2, and IL-12 between diabetic patients with different durations of the disease (P<0.05). also results showed a significant difference in the fasting glucose and Hba1c levels between diabetic patients when we used Homa-IR as independent factor (P<0.05). There was a significant difference in the fasting glucose level when diabetes also considered as a control independent factor. Also, there was significant in Hba1c level when we considered fasting glucose level as an independent factor (P<0.05) (Table 2).

**Correlation analysis:**

The correlation between the significant parameters in this work, result in a significant direct proportion between the level of IL-2 and IL-12 (P < 0.05). And both IL-2 and IL-12 were inversely proportional with fasting glucose level and Homa-IR without no significant value (P > 0.05). Also It was found that the relation between CRP with IL-2 differs from that with IL-12. The CRP was inversely proportional with IL-2 and directly proportional with IL-12. a, and these relations lack the statistical value (P > 0.05) (Table 3).

**Immunohistochemical Findings:**

Examined sections from prepared cell blocks of centrifuged whole blood samples obtained from diabetic patients revealed that about 80-85% of round cells, mostly of lymphoid origin, have positive cytoplasmic and membranous staining potency to CD8 marker in 20 HCCs (24%) (Fig.1). Meanwhile, nearly most of the examined lymphoid cells (95-100%) were negative staining for the CD4 marker. (Figs.2). Summary of the immunohistochemical findings in diabetic staining reactivity are shown in table (4) & (Fig.3).

Table 3: Correlation analysis of biomedical parameters in diabetic subjects.

| Correlation     | IL-12   |       |         |       |
|-----------------|---------|-------|---------|-------|
|                 | r       |       | p       |       |
| IL-2            | 0.87    |       | 0.048   |       |
|                 | IL-2    |       | IL-12   |       |
|                 | r       | p     | r       | p     |
| Fasting Glucose | - 0.183 | 0.172 | - 0.017 | 0.979 |
| CRP             | - 0.082 | 0.413 | 0.125   | 0.31  |
| Homa-IR         | - 0.083 | 0.36  | - 0.188 | 0.114 |

P < 0.05 is significant

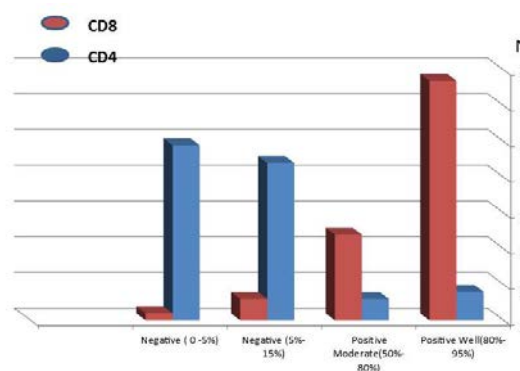


Figure 3. A chart represents the percentages of both CD4 and CD8 expression in lymphoid cells of peripheral blood of diabetic patients.

Table 4: Summary of immunohistochemical findings of CD4 & CD8 in lymphoid cells of cell block tissue of diabetic patients.

| Immunoreactivity Differentiation | CD <sup>4</sup> (81) |    | CD <sup>8</sup> (81) |    |
|----------------------------------|----------------------|----|----------------------|----|
|                                  | No.                  | %  | No.                  | %  |
| Negative (0-5%)                  | 40                   | 49 | 2                    | 2  |
| Negative (5%-15%)                | 36                   | 44 | 5                    | 6  |
| Moderate Positive (50%-80%)      | 5                    | 6  | 20                   | 24 |
| High Positive (80%-95%)          | 0                    | 0  | 55                   | 67 |

**DISCUSSION**

2DM is mostly a prevalent type of diabetes all over the world; with complications has great social and economic effects (Connolly ,2000). Many studies approved the great relationship between type-2 diabetes and innate immunity

through an inflammation mechanism. In addition, the alteration of the cytokine secretion due to type-2 diabetes is not understood until now. DM is an inflammatory disease characterized by inflammation due to the hyperglycemia and hyperlipidemia. This inflammation is mediated by several mediators, which called cytokines that have disturbance level in according to their importance and function in the inflammation process (Pickup, 2004).

Type-2 DM is associated with expanded frequencies of mycobacterial antigen-specific CD4<sup>+</sup> T helper type 1 (Th1) (Ghanashyam, 2014). Also, other investigators demonstrated that T1-DM mediates amplification of CD8<sup>+</sup> T-cells and more rapid  $\beta$ -cell loss which mean that CD4<sup>+</sup> T-cell appears to be present in both T1-DM and T2-DM while auto reactive CD8<sup>+</sup> T-cells are unique to T1-DM. Thus, the CD8<sup>+</sup> cells may serve as a more T1-DM specific biomarker. Other authors showed that those with TB-DM have elevated frequencies of interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-2 (IL-2) and IL-17F secreting CD8<sup>+</sup> T cells (Nathella *et al.*, 2015). These findings with in agreement with our biochemical and immunochemical results obtained during this study.

The increased expansion of CD8<sup>+</sup> T and NK cells expressing pro-inflammatory cytokines could be increased CD8<sup>+</sup> T and may altered subset distribution or differential proliferation. Our data suggest that it is unlikely that alterations in T or NK cell numbers or subset distribution or intrinsic proliferation status was the primary cause enhancing expansion of cytokine-producing T cells (Cullen and Martin, 2008).

Besides, the T-cell surface have molecules that involved in regulation of T-cell interleukin 2 (IL-2) responses. There are several monoclonal antibodies (mAbs), which inhibit IL-2 responses of freshly isolated CD8<sup>+</sup> T cells (Cullen and Martin, 2008). So that, our data on the lack of differentiation of CD4<sup>+</sup> antigen and increase differentiation of CD8<sup>+</sup> T strongly support the decreased IL-2 and increased IL-12 than control volunteers and this biomarker have an important role for DM.

These results showed that 80-85% of round lymphoid cells positive to CD8 marker, while most of the examined lymphoid cells (95-100%) were negative for the CD4 marker this support the work of Shingo *et al* who found that the increased number of CD8<sup>+</sup> tumor-infiltrating lymphocytes (TILs) characterized by decreased production of IL-2, TNF $\alpha$ , and IFN $\gamma$ . The CD8<sup>+</sup> TILs capable of producing many other cytokines were mainly PD-1-Tim-3+, an effector for memory cells (Takahashi *et al.*, 1992).

These results show a significant decrease in IL-2 level and an increase in IL-12 level in type-2 diabetic patients than other controls. However, the lack of the statistical power may be because of sample size, life style, age mean and many other environmental factors. They can be used as prediction and not diagnostic for type-2 diabetes.

Our results are in agreement with Shingo and his team who discussed the relation between IL-2 and diabetes in new England population and found no significant association between them, but they found an association between IL-2 and type-1 diabetes (Shingo *et al.*, 2015). This observation is supported by Memon and his team who made their study in Swedish population and they found an association between IL-2 and insulin sensitivity in Swedish-born population (Memon *et al.*, 2015). The nature of IL-2 has two functions (pro-inflammatory and anti-inflammatory) (Shachar and Karin, 2013). This defect in the insignificant decrease in its level in this result. Banchereau and his group reported that IL-2 has a great role in controlling inflammation and thus can be considered as anti-inflammatory maker (Banchereau *et al.*, 2012).

The deactivation of IL-2 makes autoimmunity due to its role in keeping T regulatory cells in the periphery blood (Setoguchi *et al.*, 2005). Besides, giving low dose of IL-2 to non-obese diabetic mice can make inhibition to the progression of diabetes and thus reduce its establish. IL-2 probably makes inhibition of diabetes by inducing its reactive CD4<sup>+</sup>Foxp3<sup>+</sup> triggering cells, which reduce the reactive effector cells (Grinberg-Bleyer *et al.*, 2010).



Once the Treg cells stimulated, it made a reduction in the number of TH17 and TFH cells, as well as IL-2 can inhibit the uncontrolled growth of immune responses and thus reduce the inflammation. These results have important implications in the treatment strategy; also, the regulatory functions of IL-2 were studied by Koreth and his team in 2011. They demonstrated that intake of low dose of IL-2 can stop inflammation and improve disease in patients with chronic graft-versus-host disease or hepatitis C virus-related vasculitis (Koreth *et al.*, 2011).

The decrease in IL-2 level in this study was supported by the result of Dwyer study who reported that IL-2 could be used as a therapy for type 1 diabetes with low doses due to its probable toxicity (Dwyer *et al.*, 2016). However, there are no previous studies cleared that IL-2 used as a therapy in case of type 2 diabetes. On other hand, other studies are not in agreement with this result and found an increase in IL-2 in type-2 diabetes They interpreted such increase on the basis that it may be due to population ethnicity, life style and other environmental factors (Penttinen, 1995).

In the present study, the level of IL-12 shows an insignificant increase and such observation was supported by the results of Momen *et al* who reported that there is no association between IL-12 and insulin sensitivity in Swedish population (Memon *et al.*, 2013). This result was confirmed by Gupta *et al* who reported that no significant association between IL-12 and type-2 diabetes (Gupta *et al.*, 2017). IL-12 is a pro-inflammatory cytokine, which act as activator for NKC, and it acts as a master controller of Th1 differentiation, and they added that the increase of IL-12 level in type-2 diabetes is theoretically logic (Adorini *et al.*, 1997). In addition, there is a great variation in IL-12 level in other different studies.

## CONCLUSION

These controversial results might be interpreted with the variation in degree of diabetes, sample size, age, environmental factors or population ethnicity. Also, the type

of treatment can affect the level of cytokines. Therefore, it can be recommended that more studies with larger sample size can give results that are more precious.

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