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RESEARCH ARTICLE

Phenotypic Analysis of Lymphocyte Populations in Type 1 Diabetes Mellitus

Ahmed Hassan[1], Mohamed Abdelal[2], Tamer Mohamed[1],Heba Assaghir[1], Hydi Ahmed[3]

1. Department of Medical Microbiology & Immunology; Faculty of Medicine, Sohag University, Sohag, Egypt.
2. Department of Pediatrics; Faculty of Medicine, Sohag University, Sohag, Egypt.
3. Ibn Sina National College for Medical Studies, Jeddah, Saudi Arabia

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Corresponding Author

Ahmed Hassan
E-mail
prof.ahmedhassan67@yahoo.com

Abstract

Type 1 Diabetes Mellitus (T1D) is a T cell-mediated autoimmune disease and is one of the most frequent chronic diseases of children and young adults. It results from immune-mediated destruction of the insulin-producing pancreatic beta cells. **Aim of the work:** This study aims to establish the phenotypic characteristics of various lymphocyte populations in type1 Diabetes Mellitus and try to correlate between the lymphocyte populations, patients' age and duration of the disease. **Patients and Methods:** The study included 30 children with type 1 Diabetes and 20 healthy control children who were tested for peripheral blood lymphocytes using flow- cytometry. The percentages of total T-lymphocytes, B-lymphocytes, helper T cells, cytotoxic T cells, activated T lymphocytes, regulatory T cells and natural killer cells were evaluated with the use of CD3, CD19, CD4, CD8, CD25, CD127, HLA-DR and CD56 monoclonal antibodies, respectively. **Results:** No significant difference was found in the percentage of different lymphocyte subpopulations in diabetic patients and controls except for T-regulatory cells that decreased significantly in patients (2.28 ± 0.37) when compared to healthy controls (3.12 ± 0.34), with a p value of 0.045 and activated cytotoxic T cells that increased significantly in diabetic patients (11.17 ± 0.73) in comparison to healthy controls (8.67 ± 1.06) with a p value of 0.02. In addition, no correlations of lymphocyte populations with patients' age and duration of the disease were found except for natural killer cells that showed significant negative correlation.

Conclusion: The differences detected in some lymphocyte subpopulations support the role of cellular autoimmune mechanisms in the pathogenesis of the disease

INTRODUCTION

A cardinal feature of the immune system is its capacity to distinguish self from nonself, a property referred to as immunological self-tolerance. Breakdown of the immune mechanisms that normally maintain self-tolerance is a factor contributing to the etiology of many human diseases, including type 1 Diabetes (T1D) **(Bellingham et al., 1953)**.

Immune tolerance is normally maintained by both central mechanisms, which are active during lymphocyte development in central lymphoid organs **(Starr et al., 2003)**, and peripheral mechanisms, which are active after lymphocyte migration to peripheral lymphoid organs. Developing T cells in the thymus undergo a selection process that purges potentially harmful T cells through a process known as negative selection. Through positive selection, the thymus promotes survival of T cells that recognize antigens in the context of the MHC molecules expressed by

the individual, a phenomenon referred to as self- MHC restriction. However, due to the delicate balance between the need for self-MHC restriction and elimination of potentially harmful T cells, a substantial proportion of T cells with low or intermediate affinity for self-antigens manage to escape to the periphery. These potentially harmful T cells are normally kept in check by a variety of peripheral tolerance mechanisms, including the induction of unresponsiveness, regulation by immunosuppressive cytokines, and suppression by T-regulatory (Treg) cells **(Mueller, 2010).**T-regulatory (Treg) and T-suppressor (Ts) cells are key players in the control of self-reactive T-cells as well as their role in the induction of peripheral tolerance. **(Sakaguchi et al., 1995).** Impairments or imbalance in the regulatory functions of the immune system appear to be important mechanisms of initiation of the autoimmune diseases **(Brusko et al., 2005).**

In T1D, as well as in other autoimmune disorders, naturally occurring Treg cells exhibit reduced suppressive properties **(Haas et al., 2005).** Another T-cell population that plays an important role in the autoimmune pathogenesis is CD8+CD28- termed T-suppressor cells. Ts cells are MHC class I restricted and operate in an antigen-dependent manner **(Cortesini et al., 2001)**. They restrain antigen-presenting cell (APC) function, and thus control further activation of T-helper cells **(Colovai et al., 2001).**

Type 1 Diabetes (T1D) is a T cell-mediated autoimmune disease involving both CD4+ T helper (Th) cells and CD8+ cytotoxic T (Tc) cells **(Liblau et al., 2002).** T1D is characterized by targeted destruction of insulin-producing pancreatic beta cells **(Viglietta et al., 2002).**T cell-mediated beta-cell destruction is induced by the release of molecules, including cytokines and chemokines **(Loetscher et al., 2001).**

The aim of this work is to determine the phenotypic characteristics of various lymphocyte populations in type1 Diabetes Mellitus, and attempt to correlate the implications of various immune cell populations in the disease process.

Patients and Methods

Patients

The study was a prospective hospital based study involving thirty children(2-16 years) previously diagnosed with type 1 Diabetes admitted to Pediatric department in Sohag University Hospital, and a control group of twenty healthy children , during the period from June 2013 to January 2014. The study protocol was approved by the local ethics committee and all patients' parents gave their consent prior to the study.

Inclusion criteria:

- Definite diagnosis of T1D according to definition of the World Health Organization criteria

Exclusion criteria:

- Children with secondary Diabetes Mellitus.
- Children with Type 2 DM.
- Evidence of active infection requiring antibiotic therapy or other concurrent diseases.
- Other autoimmune disease.
- Age less than 2 years or more than 16 years.

Methods:

All children included in the study were subjected to the following:

I. **Complete medical history.**
II. **Thorough clinical examination.**
III. **Investigations:**
    - Complete blood count
    - C reactive protein level
    - Blood glucose levels.
IV. **Flowcytometric analysis of lymphocyte populations**

Peripheral blood lymphocytes processing

Fresh 2ml ethylene diamine tetraacetic acid-anticoagulated peripheral blood samples were collected. According to manufacturer's instructions, 10 µl MoAb was added to 100 µl EDTA blood , vortexed and incubated for 15 min at room temperature in the dark. 1ml lysing reagent (VersalyseTM -Beckman Coulter) was added, immediately vortexed then incubated for 10 min at room temperature in the dark. We verified that there were no significant differences in the investigated markers levels in samples stained immediately after collection in comparison with samples stained up to a maximum 24-hours time interval post sampling and stored at 4^{o}C.

Fluorescein-conjugated monoclonal antibodies (MoAbs) were used for staining lymphocytes namely anti-CD4, anti-HLA-DR labeled with fluorescein isothiocyanate (FITC), anti-CD8, anti-CD25, anti-CD56 labeled with phycoerythrin (PE), anti-CD3, anti-CD19, labeled with electron coupled dye (ECD) and anti CD127 labeled with phycoerythrin-cyanin5 (PE– Cy™5) (Beckman Coulter, France.)

Lymphocyte subpopulations assays

The Lymphocyte subpopulations analyzed were CD3/CD4 (total T helper cells), CD4/CD25 (activated T helper cells), CD4/CD25/CD127low (T regulatory cells), CD3/CD8(total T cytotoxic cells), CD8/HLA-DR (activated T cytotoxic cells), CD4/CD45RO and CD8/CD45RO (memory T cells), CD3- CD56+ (natural killer cells), CD19 (B lymphocytes). The blood samples were analyzed with Epics-XL flowcytometer (Coulter, USA). Lymphocytes were analyzed using a gate set on forward scatter versus side scatter, with a standard 4-color filter configuration using System II software version 3.0 (Coulter, USA).

We recognized 2 different strategies of Treg identification based on surface markers: (1) gating on CD4 cells with high expression of CD25 (2) gating on CD4 cells expressing CD127 low/negative levels (CD127low) cells.

Statistical Analysis

Quantitative data were presented as Mean and Standard deviation and Correlation coefficient "r" test was used to correlate two numeric quantitative variables. For all tests P value < 0.05 was considered significant.

Results

Patients' demographics:

The study included thirty type 1 Diabetes children (13 females and 17 males) and twenty healthy children as control group 11 females and 9 males. **Table (1)**

Table (1): Demographic data of diabetic children and healthy controls

	Cases	**Controls**	**P value***
Age (years)	8.00 ± 0.70	6.36 ± 0.73	0.15
Sex: Females **Males**	13 (43.3%) 17 (56.6%)	11 (55%) 9 (45%)	0.42

Results of Flowcytometric analysis of lymphocyte populations:
The study included 30 patients and 20 healthy controls, the mean fluorescence intensity (MFI) was used to evaluate the percentage of different lymphocyte populations

Table (2): Lymphocyte populations in diabetic patients and controls.

Lymphocyte subpopulations	Cases	Controls	P value*
CD3/CD4	35.33 ± 2.46	30.47 ± 1.91	0.26
CD4+ CD25+	4.07 ± 0.47	4.19 ± 0.87	0.87
CD4 +CD25+ CD127 Low	2.28 ± 0.37	3.12 ± 0.34	**0.045**
CD8+	17.80 ± 1.43	18.99 ± 1.20	0.37
CD8 +HLA DR+	11.17 ± 0.73	8.67 ± 1.06	**0.02**
CD19+	9.89 ± 1.30	12.66 ± 2.20	0.61
CD3 -CD56+	3.47 ± 0.54	4.27 ± 0.64	0.23

Data were expressed as mean ± standard deviation.

As shown in table 2, no significant difference was found in the percentage of different lymphocyte subpopulations in diabetic patients and controls except for T-regulatory cells(CD4 +CD25+ CD127 Low) that decreased significantly in patients (2.28 ± 0.37) when compared to healthy controls (3.12 ± 0.34) with P value= 0.045 **Figure (1**).

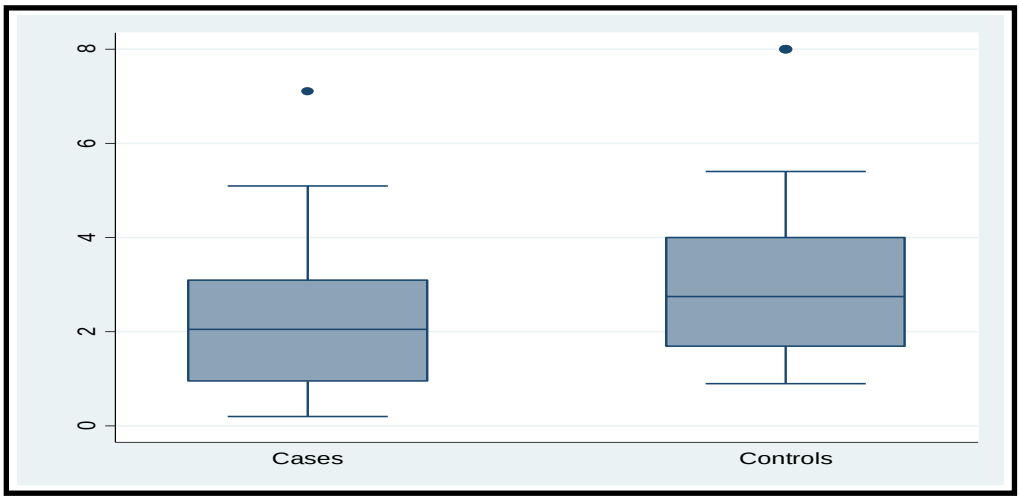

Figure (1): Comparison of T- regulatory cells in patients and controls.

There was also significant increase in the percentage of activated cytotoxic T cells (CD8+HLADR+) in diabetic patients (11.17 ± 0.73) in comparison to healthy controls(8.67 ± 1.06) with P value =0.02 **Figure (2).**

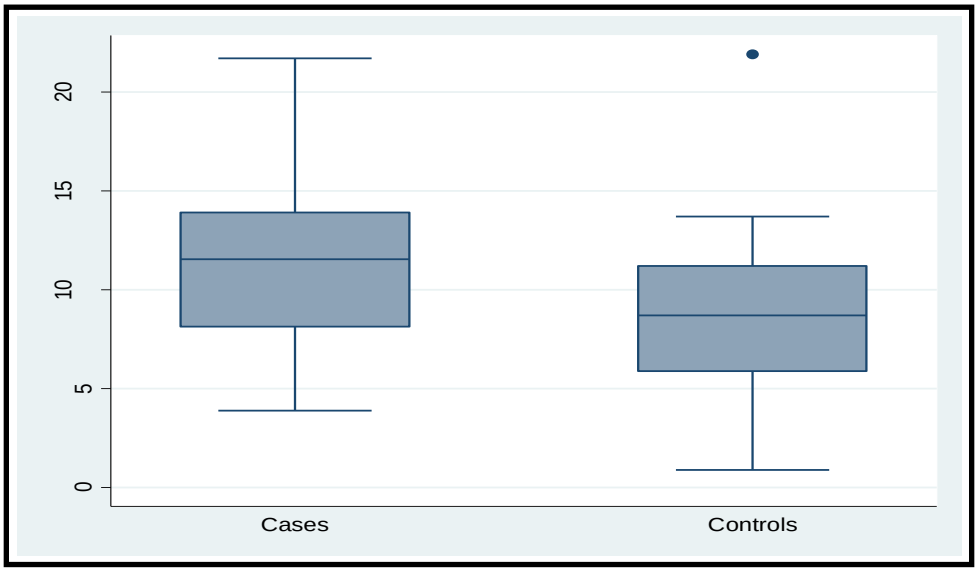

Figure (2): Comparison of activated cytotoxic T cells in patients and controls.

- **Correlations of lymphocyte populations with age** in patients was performed .The correlation coefficient, denoted by r, is a measure of the strength relationship between two variables. **Table (3).**

Table (3): Correlation between age of patients and different lymphocyte populations.

	Correlation Coefficient	**P value***
CD4+	0.12	0.52
CD4+ CD25+	-0.29	0.12
CD4+ CD25 CD127 Low	-0.15	0.44
CD8	-0.12	0.54
CD8 HLA DR	0.26	0.17
CD19	0.12	0.53
CD3 CD56	-0.49	0.006

Spearman rank correlation test was used. 0 indicates no linear relationship.
Values between 0 and 0.3 (0 and -0.3) indicate a weak positive (negative) linear relationship.
Values between 0.3 and 0.7 (0.3 and -0.7) indicate a moderate positive (negative) linear relationship.

We found that there was weak positive correlation among T helper cells (CD4+), activated T cytotoxic (CD8+HLADR), B lymphocyte (CD19+), increasing with age but without statistical significance.
Also a negative age-dependent trend within the activated T-helper (CD4+CD25+)**,** T-reg (CD4+CD25+CD127low), and T cytotoxic (CD8+) population was detected in patients with decreasing percentages of cells with increasing age; but without any statistical significance.
Natural killer cell (CD3- CD56+) percentages showed moderate negative correlation with age with statistical significance P value = 0.006. Figure (3)

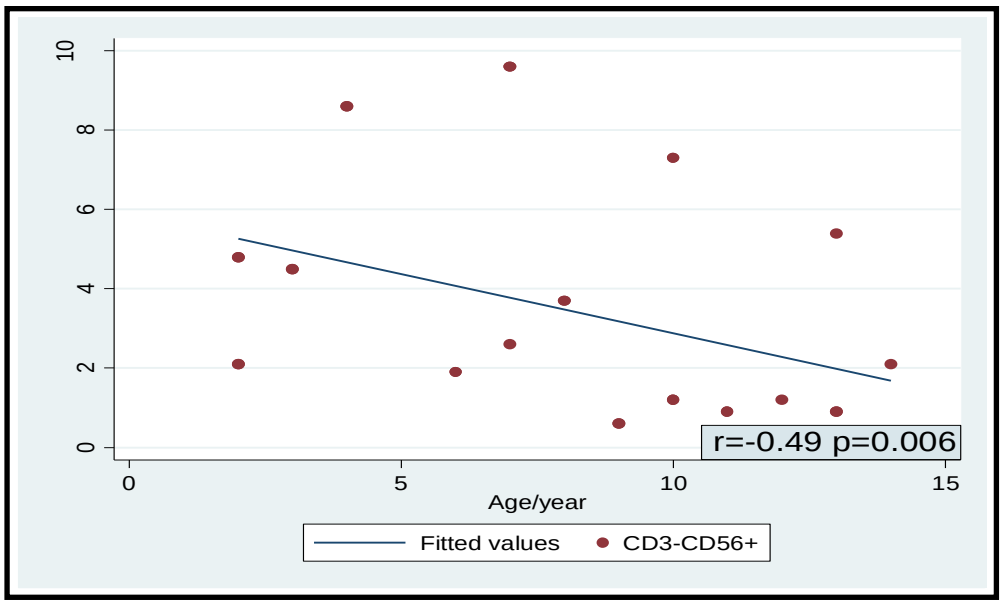

Figure (3): Correlation between patients' age and natural killer cells percentage.

- Correlations of lymphocyte populations with disease duration in patients was performed.

Table (4): Correlation between disease duration and different lymphocyte populations.

	Correlation Coefficient	P value*
CD4+	0.09	0.63
CD4+ CD25+	0.08	0.65
CD4+ CD25 CD127 Low	-0.10	0.59
CD8+	-0.18	0.34
CD8+ HLA DR+	0.20	0.28
CD19+	0.15	0.44
CD3- CD56+	-0.44	**0.02**

We found that there was weak positive correlation among T helper cells (CD4+), activated T-helper (CD4+CD25+), activated T cytotoxic (CD8 HLA DR), B lymphocyte (CD19+) increasing with disease duration but without statistical significance.
Also weak negative correlation within T-reg (CD4+CD25+CD127low), and T cytotoxic (CD8+) population was detected in patients with decreasing percentages of cells with disease duration but without any statistical significance.
Natural killer (CD3+CD56+) percentages showed moderate negative correlation with disease duration with statistical significance P value = 0.02. **Table (4).**

As regards T lymphocyte subpopulations, there was no significant difference in the percentage of (CD4+CD25+) activated T helper cells among diabetic patients and control population but (CD4+CD25+CD127low) T-regulatory lymphocytes was significantly decreased in T1D patients when compared to

healthy controls and remarkably increased frequency of activated cytotoxic (CD8+HLADR) in diabetic patients in comparison to healthy controls.

No Correlations of lymphocyte populations with age in patients and duration of the disease were found except for natural killer cells. Natural killer cells showed significant negative correlation with age and duration of the disease in diabetic children, in that natural killer cells showed decreased frequency as the age of the patients or the duration of the disease increases.

Discussion

Autoimmune diseases are characterized by multiple defects of the immune system including impaired function of regulatory mechanisms (**Dejaco et al., 2005**). We were able to describe such differences and compare various lymphocyte populations that could be directly or indirectly involved in the pathogenesis.

In our study there were no significant differences in the percentage of T helper, T cytotoxic lymphocytes, B lymphocytes, Natural killer cells among diabetic patients and controls. These results are concordant with (**Zahran et al ., 2012) .**

Hedman et al. in 2008 showed that Percentages of CD4+ T helper and CD8+ T cytotoxic cells were similar in samples from diabetic and healthy children, indicating alteration in their phenotype rather than amount of circulating cells. Interestingly, in their study a higher ratio of CD4+ and CD8+ cells was observed among T1D compared with healthy children, low Th1-associated receptor expression of CD4+ cells together with an increased amount of CD8+ cells expressing CD45RA and CCR7, and reduced chemokines secretion in the PBMC cultures in T1D patients, suggesting suboptimal Th function and impaired Tc responses in children with T1D close to the diagnoses. This could result in continuous recirculation and recruitment of Tc naive/effector cells enhancing the inflammatory state in the pancreas, while the Th1 cell population in peripheral blood is suppressed (**Hedman et al., 2008**). The presence of IL-2 receptor a-chain (CD25) on CD4+ T helper lymphocytes is expressed by antigen-activated T-lymphocytes. In our study there was no difference in the level of (CD4+CD25+) activated T helper lymphocytes in patients and controls.

Mikulkova et al. in 2010 showed higher distribution of activated T helper lymphocytes (CD4+CD25+CD127+) in T1D patients but not in healthy controls (**Mikulkova. et al., 2010**). **Brusko et al.** in 2005 reported that when the frequency of CD4+ CD25+ T-cells was plotted, type 1 diabetic patients exhibited lower frequencies than control subjects (**Brusko et al ., 2005).**

Treg population defined by low expression of IL-7 receptor (CD127) and therefore phenotype CD4+CD25+CD127 low perfectly matches this; in our study we found notably decreased numbers of T- regulatory population in diabetic patients. The MFI of (CD4+CD25+CD127low) T-regulatory population in cases is (2.28 ± 0.37**)** and in controls (3.12 ± 0.34) with P value= 0.045, this decline in patients could implies that the deficiency of regulatory T cells may has a role in the pathogenesis of type 1 Diabetes. **Zahran et al**., **2012** found lower frequency in T regulatory lymphocytes in patients than controls **(Zahran et al., 2012).** Other authors found a statistically significant decrease of T regulatory cells in children with newly diagnosed T1D **(Lucznski et al., 2009)**, (**Ryba et al., 2010**).

Glisic –Milosavljevic et al. in 2005 reported a higher level of ongoing apoptosis in T regulatory cells in recent onset T1D children and in children at high risk for the disease. On the contrary in long lasting T1D and T2D children, T regulatory cell apoptosis is the same level as in control children. This high level of T regulatory cell apoptosis could explain the decrease of regulatory T cells in our patients (**Glisic –Milosavljevic et al., 2005**).

Lindley et al. in 2005 demonstrated that rather functional defects are present in T1D T reg population. The regulatory capacity of (CD4+CD25+) T regulatory cells from patients with type 1 Diabetes was markedly diminished in comparison with control subjects in terms of both suppression of proliferation and control of secretion of the pro inflammatory cytokine IFN-γ .Conversely, the anti-inflammatory cytokine interleukin-10 was found at higher concentrations in co cultures from control subjects. IFN- γ is believed to have a central role in T-cell–mediated destruction of pancreatic B-cells, and it is known that islet-autoreactive T-cells from patients with type 1Diabetes are characterized by secretion of this cytokine, whereas cells from control individuals are characterized by secretion of interleukin-10. Although it has been demonstrated that, in vitro, suppression of CD4+CD25+ T-cells seems to be independent of interleukin- 10, secretion of this cytokine may represent a useful marker to identify effective regulation by cells in the CD4+CD25+ population. Taken together, these novel data support the proposal that the development of type 1Diabetes is accompanied by defective immune regulation by at least one of the major suppressor T-cell populations defined to date (**Lindley et al., 2005**).

In our study notably increased numbers of (CD8+HLADR) activated cytotoxic cells in diabetic patients in contrast to control subjects were found. MFI of (CD8+HLADR) activated cytotoxic in patients WAS (11.17 ± 0.73) and in controls (8.67 ± 1.06) with P value =0.02. **Mikulkova et al**. in 2010 showed higher distribution of(CD8+CD25+) activated cytotoxic lymphocytes in T1D children than healthy controls **(Mikulkova et al., 2010).**

T lymphocyte populations were assessed with regards to patients' age. No statistically significant data was obtained from these different populations. Negative correlation was found between Treg populations in diabetic patients with age. Similar to our results **Mikulkova Z et al ., 2010** showed a downward trend with age in the healthy controls group , as well as in the patients groups , in the population of T-regulatory cells **(Mikulkova et al., 2010)**. In contrast to our results **Zahran et al .** in 2012, showed that Positive correlations were observed between both age and insulin C peptide and frequency of CD4+CD25+High Foxp3. **(Zahran et al., 2012). Brusko et al.** in 2005 reported that increasing age was associated with an increase in total CD4+CD25+ frequency , however in that study, Treg-specific marker such as CD127 low for precise identification of Treg population was not used (**Brusko et al ., 2005)** . As regards (CD8+HLA-DR+) activated cytotoxic lymphocytes positive correlation with age was recorded, showing increased frequency of activated cytotoxic lymphocytes (CD8+CD25+). This is concordant with other studies **(Mikulkova Z et al., 2010)** .

Natural killer cells showed negative correlation with age and duration of the disease in diabetic children. Natural killer cells showed decreased frequency as the age of the patients or the duration of the disease increases.
Rodacki et al. in 2007 showed that the onset phase of the disease did appear to be marked by NK cell perturbations. Significant decrease in numbers of NK cells was found. Activation phenotypes were more diverse than those observed in control subjects or patients with established type 1 Diabetes, with particularly strong gamma-interferon expression, on the other hand patients with long-standing type 1 Diabetes exhibited reduced activation of NK cells, relative to control subjects or recent-onset patients(**Rodacki et al ., 2007**).
Lorini et al. found decreased NK cytotoxic activity with normal NK cell number, in patients with recently diagnosed disease (less than 1 year of disease duration) and particularly in long-standing diabetics (more than 3 years of disease duration) and he contribute that to a qualitative defect of the NK cells, or to a deficient IL-2 and/or TNF-alpha production, or to a immunomodulatory or immunosuppressing effect of insulin **(Lorini et al., 1994).**

Our study only addressed the NK compartment in blood; it remains possible that significant differences occur in the pancreas or draining lymph nodes, differences not reflected in the blood.

Alba et al. in 2008 reported significant increase of Natural Killer cells in the pancreas of the accelerated diabetic NOD RIP-IFN-b mice at the onset of Diabetes when compared with healthy transgenic ones. This increase in NK cells was not observed in the spleen or in the pancreatic lymph nodes from mice with accelerated Diabetes when compared with controls. The percentage of intra pancreatic T helper (CD4+) and T cytotoxic (CD8+) cells did not show significant differences when comparing subjects with accelerated disease to healthy mice, however the proportion of B cells (CD19+) showed a marked decrease at the onset of accelerated Diabetes when compared with healthy mice .Thus, accelerated Diabetes correlates with the percentage of NK cells and correlates inversely with the percentage of B cells in the islets. There was also increased expression of NK-attractant chemokines, CCL5 and CXCL10, and cytokines involved in NK function, IL-12 and IFN , in the target tissue of accelerated Diabetes which correlates with this NK subset increase (**Alba A et al .,2008) .**

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