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Does control of glycemia regulate immunological parameters in insulin-treated persons with type 1 diabetes?

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ABSTRACT

Aims: We investigated the relationships between control of glycemia and the frequencies of immune cell subpopulations and also the profile of circulating T cell cytokines in insulin-treated persons with type 1 diabetes (T1D).

Methods: Clinical data and blood samples were collected from two groups of persons with T1D exhibiting either adequate (AGC) or inadequate glycemic control (IGC), as well as from individuals without diabetes considered as a control group. Serum cytokine levels and immune cell subpopulation frequencies were determined.

Results: Irrespective of their capacity to control glycemia, the percentages of effector $CD4^+$ T-cells and $CD19^+$ B-cells were higher in persons with T1D than in controls, whilst monocytes were significantly more frequent in those with IGC than in controls. The overall frequencies of $CD4^+$ T-cells, $CD8^+$ T-cells and $Foxp3^+CD4^+CD25^+$ regulatory T-cells did not differ between the three groups. The serum levels of IL-2 and IFN-γ were lower in both groups with T1D compared to controls, whilst the level of IL-4 did not differ. The level of IL-10 was significantly lower in those with AGC compared to controls.

Conclusion: Our study shows that insulin treatment is associated with a Th2-biased systemic immune phenotype in persons with T1D, reflected by a high proportion of effector $CD4^+$ T cells and $CD19^+$ B cells and a down-regulation of Th1-type serum cytokines.

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1. Introduction

Type 1 diabetes (T1D) is a chronic T lymphocyte-mediated autoimmune disease that results in the destruction and loss of functional insulin-producing pancreatic islet beta cells [1]. The pathogenesis of T1D relies on the interaction between a genetic predisposition, immune system modulation, and environmental factors such as drugs, toxins, nutrients and viruses [2]. There is evidence that functional changes and alterations in lymphocyte and cytokine profiles are implicated in T1D onset and progression [3–5]. Indeed, in the early phase of the disease, T1D is characterized by islet inflammation

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with a predominance of activated immune-cell infiltration including $CD4^+$ T cells, B cells, natural killer cells, and macrophages, and a release of inflammatory mediators, especially proinflammatory T helper 1 (Th1) cell-derived cytokines, all contributing to β-cell apoptosis [6]. The pathological mechanisms that trigger the loss of self-tolerance between β-cell antigens and auto-reactive T-cells are not fully elucidated. However, it is well known that the destruction of insulin-secreting pancreatic beta cells in T1D that leads to insulin deficiency induces a set of metabolic disorders including hyperglycemia and dyslipidemia.

Chronic hyperglycemia has been shown to enhance proinflammatory cytokines production and to increase susceptibility to infections related to impairment of the innate immune cell functions such as monocytes, macrophages and polymorphonuclear neutrophils [7–12]. It is also associated with the development of several micro and macrovascular complications due to a proinflammatory state and to various biochemical mechanisms, leading to overproduction of reactive oxygen species and tissue damage [13,14].

The benefits of insulin therapy for glycemic control in delaying or reducing long-term T1D complications, morbidity and mortality, are well-documented [15,16]. In fact, insulin has been shown to exert its therapeutic effects by improving the anabolism of glucose, proteins and lipids, thereby preventing the deleterious effects of hyperglycemia. Thus, insulin therapy for T1D promotes an anti-inflammatory state through the reduction of proinflammatory cytokines and reactive oxygen species, and conversely, the increase of anti-inflammatory cytokines [17–20]. Moreover, many in-vitro studies have shown that insulin can induce a systemic anti-inflammatory response via modulation of proliferation, differentiation, metabolism and immune functions of neutrophils, monocytes, macrophages, effector and regulatory T cells [20–22]. However, it remains unclear whether glycemic control can modulate, in-vivo, the pattern of immune cell subtypes of T1D patients treated with insulin.

Thus, the aim of the present study was to investigate the effect of glycemic control on the frequencies of leucocytes subpopulations along with the profile of systemic T-cell derived cytokines in insulin-treated persons with T1D.

2. Material and methods

2.1. Subjects and samples

Sixty-five individulas with T1D, aged 12–37 years (mean age = 22.8 ± 7.3 years), were recruited at the principal Insulin Centre of Cotonou (Benin) by specialist clinicians. They were all long-established diabetes patients (disease duration = 7.7 ± 5.6 years) diagnosed according to the criteria of the American Diabetes Association [23] and were all on insulin treatment. After firstly analyzing biochemical data, we were able to classify persons with T1D into two metabolic groups with respect to glycemic control: thirty-four exhibited inadequate glycemic control (IGC), determined by glycated hemoglobin (HbA1c) equal to or higher than 7.0% and thirty-one showed adequate glycemic control (AGC) (HbA1c < 7.0%) [23,24]. Thirty-six control subjects were recruited and matched for age, sex, body mass index (BMI), and geographic origin of those with T1D.

Control individuals had no family history of diabetes and showed normal fasting glycaemia measured at inclusion. Persons with T1D with neurological or micro- and macrovascular complications were excluded from this study. All participants were non-smokers, had no immunosuppressive treatment, no significant history of other illness, were not pregnant, and had no clinical signs of infectious disease. All tested negative for HIV, hepatitis B, hepatitis C, and *Plasmodium falciparum* (malaria parasite) infection at inclusion.

The study was carried out in accordance with the Declaration of Helsinki (1964, as revised in Edinburgh 2000). The study was approved by the Ethical Committee on Research of the Institute of Applied Biomedical Sciences of Cotonou, Benin under the number Dec.n°075/CER/ISBA-2015. Subjects were informed of the study aim and their written consents were obtained. The privacy rights of human subjects were observed. Blood samples were collected and treated as we previously described [25,26].

2.2. Immune cell phenotyping

Innate and adaptive immune cell characterization was performed as we previously described [25,26]. Briefly, the following combinations of monoclonal antibodies (mAbs) purchased from BD Pharmingen (France) were used to measure the frequencies of cells in whole blood: anti-CD3-FITC/anti-CD4-PerCP/anti-CD8-PE/anti-CD25-PE/anti-CD127-FITC/anti-Foxp3-APC were used for T cell subset labeling, anti-CD19-FITC for B lymphocytes, anti-CD16-PE/anti-CD56-APC for NK cells, anti-CD14-FITC for monocytes, according to the manufacturer's instructions. The stained cells were acquired using a FACS Calibur flow cytometer and analyzed using FlowJo 7.6 software (BD Pharmingen, France). Gating strategies for immune cell phenotyping are shown in Fig. 1a–e.

2.3. Determination of biochemical parameters and serum cytokine concentrations

Plasma glucose, HbA1c, serum triglycerides, total and HDL-cholesterol concentrations were determined as we previously described [26]. Cytokines were quantified in serum samples by ELISA, using human Th1/Th2 ELISA MAX™ Deluxe set kits (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions. The minimum detectable concentrations were 4 pg/mL (standard ranges = 7.8–500 pg/mL) for IL-2 and IFN-γ and 2 pg/mL (standard ranges = 3,9–250 pg/mL) for IL-4 and IL-10.

2.4. Statistical analyses

Data analyses were performed using Graph Pad Prism 6.0 (Graph Pad Inc, CA, USA). The Kruskal–Wallis test, followed by Dunn's multiple comparison test, was used to analyze differences between the three groups (those with T1D exhibiting AGC and IGC and the control group). The Mann–Whitney *U* test was also used when appropriate. p values < 0.05 were considered to indicate statistically significant differences.

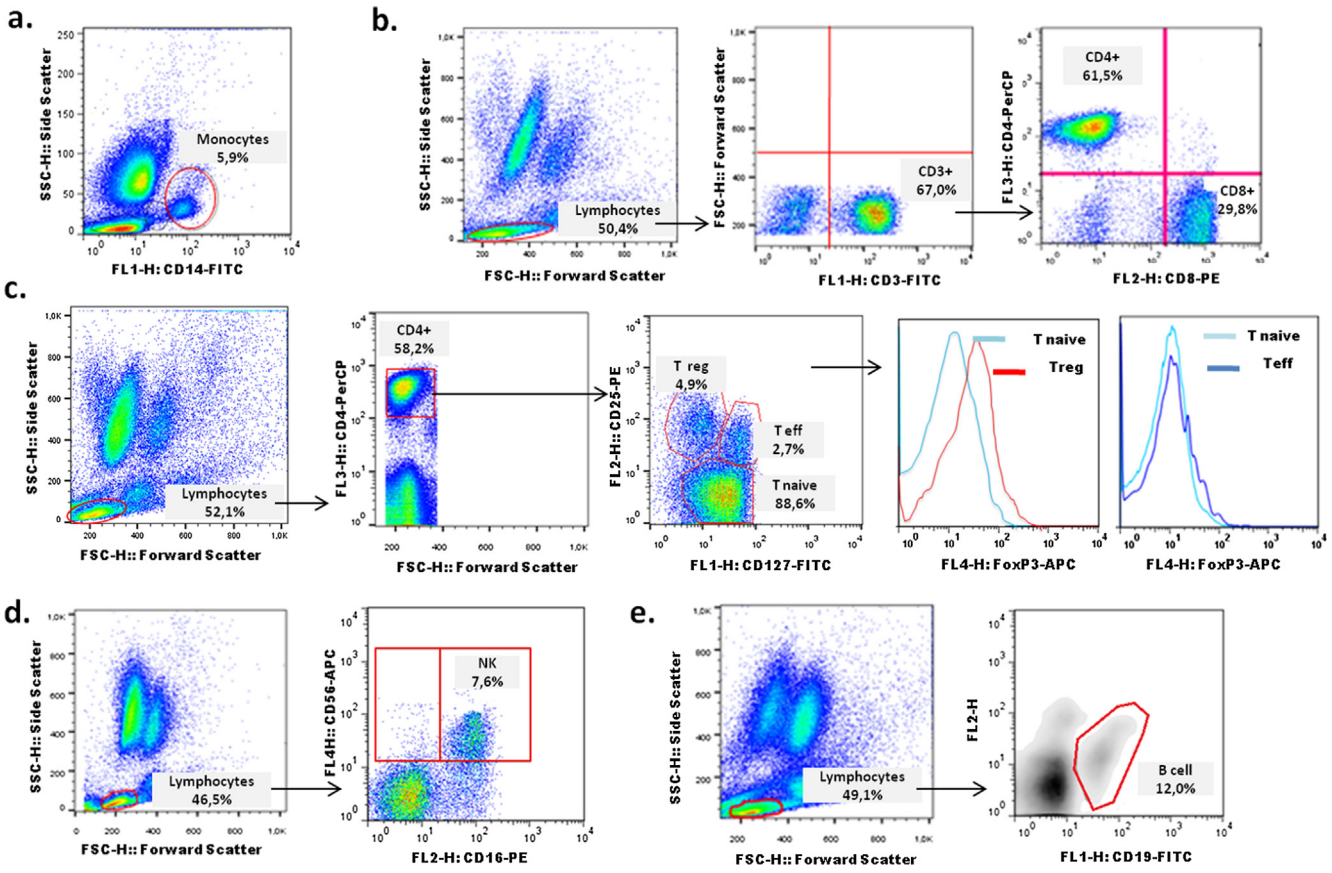

Fig. 1 – Cytometry-based gating for definition of immune cell phenotypes. (a) Monocytes ($CD14^+$) were gated from mononuclear population in blood cells. (b) $CD4^+$ and $CD8^+$ T cells were gated from $CD3^+$ lymphocytes. (c) Gating strategies for regulatory T cells (Treg: $CD4^+CD25^+CD127^-$) and effector T cells (Teff: $CD4^+CD25^+CD127^+$) and relative Foxp3 expression level determined as a function of Foxp3 expression by naïve $CD4^+$ T cells. (d) NK cells ($CD16^+CD56^+$) and (e) B cells ($CD19^+$) were gated from the total lymphocytes.

3. Results

3.1. *Anthropometric and biochemical profiles*

The anthropometric and clinical characteristics of the T1D and control groups are shown in Table 1. There was no significant difference in age or body mass index (BMI) between the T1D and control groups (Table 1). In this study, thirty-four persons with T1D exhibited inadequate glycemic control (IGC) determined by glycated hemoglobin (HbA1c) equal or higher than 7.0% and thirty-one persons with T1D showed adequate glycemic control (HbA1c < 7.0%) [23,24]. As expected, fasting glucose levels were higher in the IGC compared to either the AGC (13.5 ± 2.0 mM vs 4.8 ± 1.6 mM, $p = 0.003$) or control groups (13.5 ± 2.0 mM vs 4.0 ± 0.1 mM, $p = 0.0001$) (Table 1). Although triglyceride levels were normal, both groups with T1D showed higher levels of triglycerides as compared to control subjects. Total cholesterol and HDL-cholesterol did not differ between the groups (Table 1).

3.2. *Immune cell profiles in insulin-treated T1D patients and control subjects*

The proportions of effector $CD4^+$ T cells (T helper cells, $CD4^+$-$CD25^+CD127^+$), B cells ($CD19^+$) and monocytes ($CD14^+$) were significantly higher in the IGC group compared to controls (17.6 ± 3.5% vs 13.5 ± 1.2%, $p = 0.01$; 11.9 ± 3.9% vs 7.7 ± 2.0%, $p = 0.04$ and 9.0 ± 2.1% vs 5.8 ± 2.4%, $p = 0.03$ respectively) (Fig. 2c, e and g). The frequencies of effector $CD4^+$ T cells and B cells, but not those of monocytes, were also significantly higher in the AGC group compared to controls (19.9 ± 1.4% vs 13.5 ± 1.2%, $p = 0.004$ and 12.3 ± 2.7% vs 7.7 ± 2.0%, $p = 0.02$ respectively) (Fig. 2c and e). On the other hand, there was no significant difference in the frequencies of effector $CD4^+$ T cells, B cells and monocytes between those with AGC or IGC (Fig. 2c, e and g). The proportions of $CD4^+$ T cells, $CD8^+$ T cells, regulatory T cells ($CD4^+CD25^+CD127^-Foxp3^+$) and natural killer cells did not differ between the groups with T1D and controls (Fig. 2a, b, d and f).

Table 1 – Anthropometric and biochemical data of participants.

	Control subjects (Ctrl)	T1D with AGC	T1D with IGC	P value Ctrl vs AGC	P value AGC vs IGC	P value Ctrl vs IGC
Number	36	31	34	–	–	–
Male/Female ratio	18/18	14/17	15/19	–	–	–
Age (years)	24.9 ± 1.7	25.5 ± 5.3	21.7 ± 1.5	NS	NS	NS
BMI (kg/m^2)	24.3 ± 1.9	21.2 ± 2.7	24.3 ± 2.9	NS	NS	NS
Duration of disease (years)	–	9.0 ± 4.3	6.6 ± 1.1	–	NS	–
Fasting glucose (mM)	4.0 ± 0.1	4.8 ± 1.6	13.5 ± 2.0	NS	0.003	0.0001
HbA1C (%) (mmol/mol)	4.7 ± 0.2 28 ± 2	5.9 ± 0.4 41 ± 5	10.1 ± 0.6 87 ± 7	0.04	0.0007	0.0001
Urea (mM)	2.8 ± 0.2	3.0 ± 0.3	3.9 ± 0.4	NS	NS	0.01
Creatinemia (μM)	64.1 ± 3.46	77.6 ± 10.5	67.3 ± 3.9	NS	NS	NS
Total Cholesterol (mM)	3.9 ± 0.30	4.0 ± 0.5	4.1 ± 0.3	NS	NS	NS
HDL-cholesterol (mM)	1.60 ± 0.18	1.5 ± 0.2	1.4 ± 0.1	NS	NS	NS
Triglycerides (mM)	0.76 ± 0.07	1.1 ± 0.1	1.3 ± 0.2	0.02	NS	0.01

BMI: Body Mass Index; HDL: High Density Lipoproteins; HbA1c: glycosylated hemoglobin. Values are means ± SEM. n = 36 control subjects as group without diabetes (Ctrl), n = 31 persons with T1D showing adequate glycemic control (AGC) and n = 34 persons with T1D showing inadequate glycemic control (IGC). NS = no significant difference. Ranges of normal values: Fasting glucose: 3.88–6.10 mM; Total cholesterol: 3.23–6.46 mM; HDL-cholesterol: 1.16–1.94 mM; Triglycerides: 0.57–1.71 mM; Urea: 2.50–7.49 mM; Creatinine: 53.04–123.76 μM; HbA1c: 4–6%.

3.3. *Profile of serum cytokines in insulin-treated T1D patients and control subjects*

Serum IL-2, IL-10 and IFN-γ levels were significantly lower in the AGC group compared to controls (6.7 ± 2.4 pg/mL vs 23.1 ± 4.4 pg/mL, p = 0.013; 4.9 ± 1.6 pg/mL vs 11.8 ± 2.5 pg/mL, p = 0.03 and 14.4 ± 2.9 pg/mL vs 82.7 ± 33.8 pg/mL, p = 0.029 respectively) whilst the concentration of IL-4 did not differ between the groups (Table 2). Serum IL-2 and IFN-γ levels were also significantly lower in the IGC group compared to controls (7.0 ± 1.5 pg/mL vs 23.1 ± 4.4 pg/mL, p = 0.038 and 28.7 ± 6.3 pg/mL vs 82.7 ± 33.8 pg/mL, p = 0.033 respectively), whilst IL-4 and IL-10 levels did not differ between the groups. There was no significant difference in IL-2, IL-4 and IL-10 levels between the two T1D groups, however, serum IFN-γ levels were higher in those with IGC compared to those with AGC (28.7 ± 6.3 pg/mL vs 14.4 ± 2.9 pg/mL, p = 0.045) (Table 2). The ratios of cytokine levels (IL-2/IL-4 and IFN-γ/IL-4) displayed a trend towards a down-regulation of Th1 cytokines (IL-2 and IFN-γ) in those with T1D as compared to controls (Table 2).

4. Discussion

The beneficial effect of insulin therapy through regulation of metabolic mechanisms and immune responses has been shown to improve glycemic control and reduce the risk of long-term complications in persons with T1D [14–16,20,27]. It has been reported that glycemic control may modulate the immune responses of peripheral blood mononuclear cells in T1D patients displayed after in-vitro stimulation [21,28–30]. However, whether glycemic control can modulate the profiles in-vivo of immune cell subtypes of those with T1D treated with insulin has not been determined.

In the present study, we investigated the effect of glycemic control on the in-vivo pattern of a wide array of immune parameters (innate and adaptive immune cells and cytokines) in insulin-treated persons with T1D. We observed that the frequencies both of effector CD4$^+$ T cells and of CD19$^+$ B cells were higher in persons with T1D than in controls, regardless of their capacity to control glycemia, suggesting that blood sugar levels do not influence the proportion of these cells in those with T1D. This idea is consistent with the results of previous studies that demonstrated that lymphocyte proliferation was increased in those with diabetes, irrespective of glycemic control [21]. Furthermore, it has been previously reported that insulin treatment had no effect on CD4$^+$ T cell proliferation [31].

In agreement with previous studies [32–36], no significant differences were observed in the frequencies of NK cells, CD4$^+$ T cells and regulatory T cells (Foxp3$^+$ T cells) between those with T1D and matched controls. However, the fact that the frequencies of effector CD4$^+$ T cells significantly increased while those of regulatory T cells did not change in those with T1D compared to controls, irrespective of their glycemic control, may probably be explained by the general impairment of the immune-suppressive activity of regulatory T cells that has been described in those with T1D [37,38].

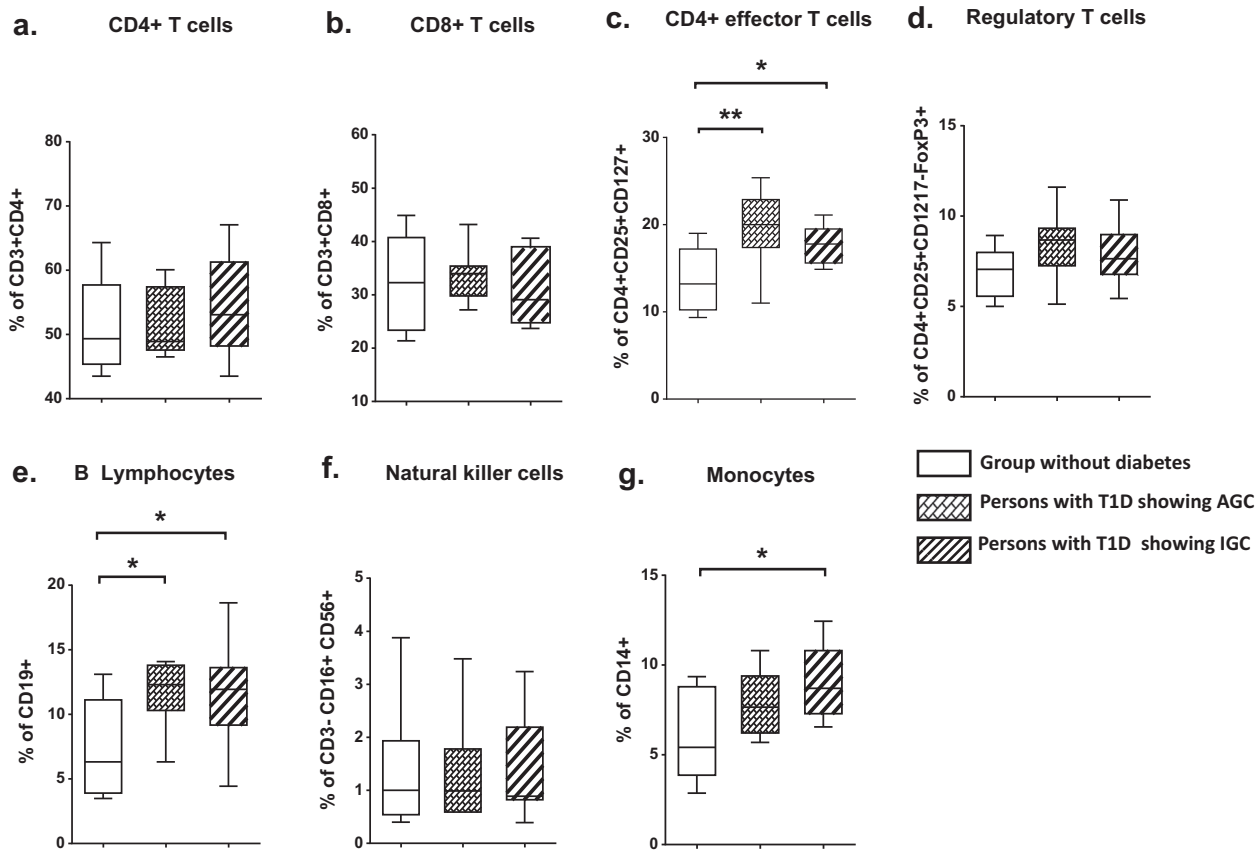

Fig. 2 – Frequencies of peripheral blood immune cell subsets in T1D and control groups. Data shown as box plots representing medians with 25th and 75th percentiles and whiskers illustrating 10th and 90th percentiles of immune cell subset frequencies: (a) $CD4^+$ T cells, (b) $CD8^+$ T cells, (c) effector T cells ($CD4^+CD25^+CD127^+$), (d) regulatory T cells ($CD4^+CD25^+CD127^-FoxP3^+$), (e) $CD19^+$ B lymphocytes, (f) Natural killer cells and (g) Monocytes from n = 36 control subjects as group without diabetes, 31 persons with T1D showing adequate glycemic control (AGC) and 34 persons with T1D showing inadequate glycemic control (IGC). The statistical differences were performed using the nonparametric Kruskal-Wallis test. $^*p < 0.05$; $^{**}p < 0.01$.

Clinical and experimental studies support the notion that T1D is a chronic Th1-mediated autoimmune disease, especially at its onset [39]. In the present study, the observed down-regulation of IL-2 and IFN-γ in T1D patients is in agreement with previous studies that provided evidence against a Th1 paradigm in T1D onset and progression [39–43]. Independently of glycemic control, we observed that the proportion of $CD19^+$ B cells was increased while Th1 cytokines (IL-2 and IFN-γ) were down-regulated in T1D persons compared with control subjects. Such observations may clearly be related to the insulin treatment in these patients. Indeed, several studies have documented the anti-inflammatory properties of insulin through downregulation of pro-inflammatory cytokines and promoting the polarization of effector $CD4^+$ T cells towards a Th2-type response [20,22,26,31,44]. We observed here also that the IL2/IL-4 and IFN-γ/IL-4 (Th1/Th2) ratios were shifted in favor of IL-4 in those with T1D, regardless of glycemic control. Taken together, these data were consistent with a humoral immune response reflected by the activation and expansion of $CD19^+$ B lymphocytes [45,46] observed in insulin-treated persons with T1D.

In the current study, the high frequencies of $CD14^+$ monocytes in those with IGC was in accordance with previous studies suggesting that activation and expansion of monocytes was related to hyperglycemia in these patients and to the risk of developing vascular complications [9,47]. On the other hand, the proportion of monocytes was not higher in those with AGC suggesting that glycemic control may be involved in reducing the expansion and/or deleterious proinflammatory activity of monocytes in these patients, possibly due to the anti-inflammatory effects of insulin on monocytes [14,20,22].

As far as the IL-10 levels are concerned, there was no difference between the groups with T1D, irrespective of glycemic control, and the control group without diabetes. The present data support previous studies that showed that glycemic control did not influence IL-10 produced in cultured peripheral blood mononuclear cells from those with diabetes [28,29]. Furthermore, those with AGC had completely normalized their glycemia but their serum level of IL-10 was even lower than in the controls. This is in agreement with a previous report that suggested that insu-

Table 2 – Serum Th1 (IL-2, IFN-γ), Th2 (IL-4) and IL-10 cytokine levels in persons with T1D and control subjects.

	Control subjects (Ctrl)	T1D with AGC	T1D with IGC	P value Ctrl vs AGC	P value AGC vs IGC	P value Ctrl vs IGC
IFN-γ (pg/mL)	82.7 ± 33.8	14.4 ± 2.9	28.7 ± 6.3	0.029	0.045	0.033
IL-2 (pg/mL)	23.1 ± 4.4	6.7 ± 2.4	7.0 ± 1.5	0.013	NS	0.038
IL-4 (pg/mL)	4.5 ± 1.6	2.1 ± 0.3	2.5. ± 0.4	NS	NS	NS
IL-10 (pg/mL)	11.8 ± 2.5	4.9 ± 1.6	8.4 ± 3.3	0.03	NS	NS
IL-2/IL-4 (pg/mL)	5.13	3.19	2.8			
IFN-γ/IL-4 (pg/mL)	18.38	6.86	11.48			

Values are means ± SEM. n = 36 control subjects as group without diabetes (Ctrl); n = 31 persons with T1D showing adequate glycemic control (AGC); and n = 34 persons with T1D showing inadequate glycemic control (IGC). NS = no significant difference.

linemia was associated with a reduced systemic level of IL-10 [48].

As far as the clinical implication of serum T-cell cytokines and immune cell subpopulation frequencies in T1D is concerned, it is well admitted that metabolic disturbance and altered immune parameters such as cytokines and immune cells in the peripheral blood of those with T1D are involved in the onset and the progression of T1D complications [3–5,15]. Among these complications, the higher susceptibility of persons with diabetes to infections has been reported and has been linked to their defective immune response [7–10,28,41,49]. In the present study, insulin treatment in T1D was associated with an anti-inflammatory Th2 profile in-vivo, associated with high frequencies of effector T cells and B cells and low levels of Th1 cytokines, suggesting activation and maintenance of humoral immune responses in those with T1D. This may offer a degree of protection against certain extracellular infections and inflammatory diseases. Be that as it may, the clinical implication of our findings merits further exploration in new studies.

5. Conclusion

Inadequate glycemic control was associated with a high proportion of monocytes in the peripheral blood of insulin-treated persons with T1D. Moreover, we observed that glycemic control did not influence the polarisation of effector T cells in insulin-treated persons with T1D. Indeed, these patients displayed a Th2-biased immune phenotype with a high proportion of effector $CD4^+$ T cells and $CD19^+$ B cells, and a down-regulation of Th1 serum cytokines, irrespective of their capacity for glycemic control. Further studies are required to determine whether the pattern of immunological parameters in peripheral blood of insulin-treated T1D individuals is related to the anti-inflammatory effects of the insulin treatment, and whether it can influence the immune response of such individuals against infections.

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Declaration of Competing Interest

All of the authors have nothing to declare as far as the conflict of interest is concerned.

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Authors contributions

MPN was in charge of major parts of technical aspects of work and wrote the manuscript. RF and AF participated in the technical work and contributed to the collection of blood. EKA contributed to the development of protocol and participated in the interpretation of data. KM and DH participated in the manuscript writing. AY designed the study, supervised the work, participated in the manuscript writing and established the collaborative aspects. All authors read and approved the final manuscript.

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