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Short communication

Increased serum levels of novel T cell cytokines IL-33, IL-9 and IL-17 in subjects with type-1 diabetes

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ABSTRACT

The role of adaptive immune cytokines in the pathogenesis of type-1 Diabetes Mellitus (T1DM) is well known. Even though reports on the serum levels of both Th-1 and Th-2 cytokines are available, those on newly described T cell cytokines such as IL-17, IL-33 and IL-9 in T1DM are scarce. We therefore measured the serum levels of both T cell polarizing (IL-33 and IL-12) and T cell effector (IFN-γ, IL-4, IL-10, IL-17 and IL-9) cytokines in T1DM subjects with and without microvascular (retinopathy and nephropathy) complications (MVC). All the tested cytokines were significantly elevated in T1DM subjects except for IFN-γ (which failed to attain statistical significance) with no significant difference between those with and without MVC. From the serum cytokine analysis, no apparent Th polarization could be determined for the T1DM subjects.

1. Introduction

Type-1 Diabetes Mellitus (T1DM) is an autoimmune disorder which arises due to immune mediated destruction of pancreatic β-cells [1]. Infiltration of T cells is widely believed to mediate this destruction and is facilitated by the secretion of cytokines [1]. Adaptive immune cytokines include both Th polarizing and T cell effector cytokines which together shape the adaptive arm of the immune response [2]. While the former is largely secreted by professional antigen presenting cells (APCs) and act on naïve T cells, the latter is predominantly secreted by polarized T cells and act on other immune/non-immune cells [2]. Earlier reports, including ours have shown significantly increased levels of pro-inflammatory cytokines like TNF-α, IL-6, IL-1β and GM-CSF in T1DM subjects [3]. However, reports documenting the levels of adaptive immune cytokines in T1DM are scant. Recently, several novel T cells cytokines like IL-33, IL-17 and IL-9 have been described. While IL-12 has long been known as the master regulator of Th-1 polarization, IL-33 has recently emerged as a master regulator for Th-2 polarization [4]. IL-17 and IL-9 are secreted by the newly discovered Th17 and Th9 cells, which play an important role in neutrophil recruitment and mucosal immunity respectively [5]. Even though, the involvement of these cytokines in T1DM has been implicated in animal models [6], a complete profile of adaptive immune cytokines including the recently described ones in T1DM is largely lacking. These cytokines play an important role both during early (beta cell loss) [1] and late (endothelial dysfunction) stages of the disease as shown in animal studies [1]. In the present study, we measured the serum levels of both T cell polarizing (IL-33 and IL-12) and T cell effector (IFN-γ, IL-4, IL-10, IL-17 and IL-9) cytokines in T1DM subjects with and without microvascular (retinopathy and nephropathy) complications.

2. Materials and methods

2.1. Study groups and sample size calculation

The control normal glucose tolerant group (NGT; n = 76) was obtained from the Chennai Urban Rural Epidemiological Study (CURES) [7]. Test groups include T1DM without microvascular complications (T1DM, n = 29) and T1DM with MVC (T1DM-MVC, n = 96) were obtained from our centre (2008–2010). Institutional Ethics Committee approval was obtained from the Madras Diabetes Research Foundation (Ref. No.MDRF-EC/SOC/2009/05) and written informed consent was obtained from all the study participants. The study was conducted as per principles of the declaration of Helsinki as revised in 2008. As a pilot study, cytokine levels were analyzed in 20 normal glucose tolerant (NGT) individuals, 20 T1DM

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subjects with MVC and 20 without MVC. The preliminary results obtained gave a sample size of 130 study subjects (50 NGT, 30 T1DM and 50 T1DM-MVC subjects) with p value <0.05, confidence interval of 95% and a power of 80%. We increased the sample size by 20–30% in the NGT and T1DM-MVC groups to account for the wide variation seen in serum cytokine levels.

2.2. Diagnosis of type-1 diabetes and microvascular complications

Diagnosis of T1DM was based on abrupt onset of diabetes, absence f pancreatic beta cell reserve (as shown by fasting and stimulated C-peptide levels <0.6 pmol/ml) and requiring insulin right from time of diagnosis [8]. The T1DM group with MVC included subjects with retinopathy (DR) (41.57%), nephropathy (DN) (n = 57.30%) and those with both (DN + DR) (n = 1.13%). Patients with gestational diabetes, type-2 diabetes, type-1 diabetes with macrovascular complications, renal diseases, chronic infections, liver cirrhosis, congestive heart failure and chronic lung disease were excluded. Diagnosis of microalbuminuria was performed based on albumin excretion as mentioned previously [7]. Retinopathy was diagnosed by the presence of at least one microaneurysm in the retinal photographs taken using a Zeiss retinal camera (Oberkochen, Germany). The photographs were studied according to the Early Treatment Diabetic Retinopathy study (ETDRS) criteria [7].

2.3. Anthropometric measurements and biochemical tests

Body mass index (BMI) was obtained using standard methods. Blood pressure was recorded using a sphygmomanometer (Deluxe BP apparatus; Diamond, Pune, India). Fasting plasma glucose was measured using hexokinase method, serum cholesterol by cholesterol oxidase–peroxidase–amidopyrine method, serum triglycerides by glycerol phosphate oxidase–peroxidase–amidopyrine method, high-density lipoprotein (HDL) cholesterol by direct method using polyethylene glycol-pretreated enzymes, and creatinine by Jaffe's method using a Hitachi- 912Autoanalyzer (Hitachi, Mannheim, Germany). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. Glycated hemoglobin (HbA1c), was measured by high pressure liquid chromatography (HPLC) (Bio-Rad, Hercules, CA).

2.4. Measurement of serum cytokine levels

The serum levels of IL-12 (Invitrogen, USA), IFN-γ (Invitrogen, USA), IL-4 (Invitrogen, USA), IL-33 (Biolegend, CA15-), IL-17 (PeproTech, USA) and IL-9 (ebiosciences, USA) were measured by sandwich ELISA following kit protocol. The lowest detection limits for the tested cytokines were: IL-12-1.95 pg/ml, IFN-γ-1.17 pg/ml, IL-10-9.76 pg/ml, IL-33-0.016 ng/ml, IL-4-1.9 pg/ml, IL-9-1.95 pg/ml and IL-17-0.02 ng/ml.

2.5. Statistical analysis

Data are expressed as mean values for biochemical parameters and geometric mean values for cytokines. One-way ANOVA was used to compare groups for continuous variables, whereas χ^2 test or Fisher exact test (as appropriate) was used to compare proportions. Kruskal-Wallis test was used for parameters which did not show normal distribution. Multiple comparisons were corrected using the Holm's correction for each set of analysis. All the analyses were done using SPSS statistical package (Version 20.0; SPSS, Chicago, IL) and p value less than 0.05 was considered significant.

3. Results and discussion

As shown in Table 1, a significant difference was found with respect to age across the groups, T1DM without MVC being significantly younger compared to control and TDM with MVC. BMI was significantly lower in T1DM subjects compared to the NGT group. Systolic and diastolic blood pressures were comparable between groups and were within the normal range. Total cholesterol, triglyceride lipids (TGL) and low-density lipoprotein cholesterol (LDL) were significantly lower, while the HDL levels were significantly higher in T1DM (both with and without MVC) subjects compared to NGT. Blood urea and microalbuminuria levels were significantly increased in T1DM with MVC compared to NGT and T1DM without MVC. All T1DM subjects were under insulin and anti-hypertensive drugs.

Serum IL-12 levels [Geometric mean (Range); p value] were significantly elevated in T1DM subjects with MVC [40.4(3.3–340.6) pg/ml; p < 0.001] and those without MVC [41.8(3.9–348.1) pg/ml; p < 0.001] when compared with NGT [10.89(3.3–52.0) pg/ml] (Fig. 1a). Serum IL-33 levels were also significantly elevated in T1DM subjects with MVC [0.27(0.01–10.5) ng/ml; p < 0.001] and those without MVC [0.35(0.02–10.3) ng/ml; p < 0.001] when compared with NGT [0.05(0.01–7.9) ng/ml] (Fig. 1b). Even though IFN-γ levels were slightly elevated in T1DM subjects with MVC [2.7 (2.0–89.2) pg/ml] and without MVC [3.15(2.0–202.6) pg/ml] but it was not statistically significant when compared with NGT [2.5 (2.08–13.8) pg/ml] (Fig. 1c). Serum IL-4 levels were significantly increased in T1DM subjects with MVC [7.22(3.7–738.1) pg/ml;

Table 1
Clinical characteristic of the study groups.

Clinical characteristics	NGT n = 76	T1DM n = 29	T1DM-MVC n = 96
Age (years)	32.0 ± 1.7	21.5 ± 11.0**	29.0 ± 15.2
Gender (F/M)	65/31	14/15	43/53
BMI (kg/m^2)	23.1 ± 4.3	20.3 ± 4.6*	20.3 ± 5.2**
Systolic BP (mm Hg)	114 ± 15.5	111.9 ± 14.9	113.5 ± 14.9
Diastolic BP (mm Hg)	72.8 ± 10.9	70.6 ± 7.1	72.5 ± 7.8
FPG (mg/dL)	85.9 ± 9.5	200.9 ± 110.5***	193.5 ± 104.7***
PPBS (mg/dL)	100.4 ± 18.2	251 ± 124.6***	272.8 ± 136.1***
HbA1c level (%)	5.4 ± 0.4	9.0 ± 2.3***	8.9 ± 1.9***
Serum cholesterol (mg/dL)	171.2 ± 33.0	156.8 ± 33.5	165.3 ± 36.2
Serum triglycerides (mg/dL)	114.3 ± 56.9	96.1 ± 43.5	84.2 ± 38.4***
HDL cholesterol (mg/dL)	43.1 ± 10.0	46.3 ± 11.0	48.6 ± 54.1***
LDL cholesterol (mg/dL)	102.1 ± 28.1	89.4 ± 28.3	95.5 ± 36.1
Blood urea (mg/dL)	16.2 ± 2.1	15.9 ± 2.8	23.8 ± 7.1***
Microalbuminuria (mg/dL)	7.6 ± 5.4	6.8 ± 6.2	27.6 ± 49.7***
Disease duration[a] (years)	0	8.0 ± 6.0	13.1 ± 8.7
Treatment	0	Insulin and anti-hypertensive drugs	Insulin and anti-hypertensive drugs

FPS- Fasting Plasma Glucose; PPBS- Post Prandial Blood Sugar; MVC- Microvascular Complications; HbA1c- Glycated Hemoglobin; HDL- High Density Lipoprotein; LDL- Low Density Lipoprotein; NS- Non-Significant.
T1DM and T1DM-MVC groups were statistically compared with NGT using One-way ANOVA test on PRISM software. Data are mentioned above as mean ± SD for continuous variables and proportions are mentioned as percentage.
[a] Comparison between T1DM and T1DM-MVC groups using Mann-Whitney test.
* <0.05.
** <0.01.
*** <0.001.

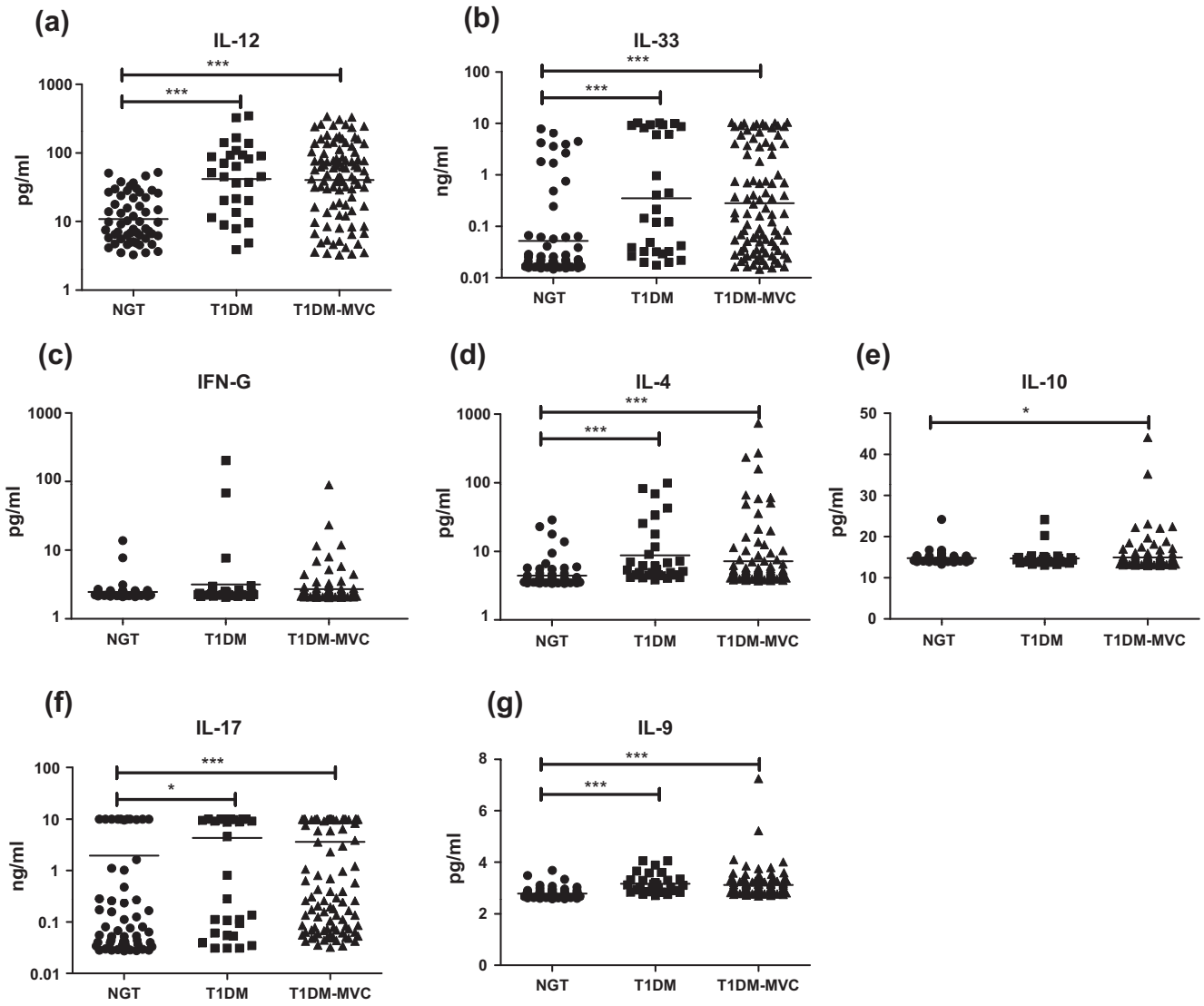

Fig. 1. Serum levels of Interleukin (IL)-12 (a), IL-33 (b), IFN-γ (c), IL-4 (d), IL-10 (e), IL-17 (f) and IL-9 (g) in NGT, T1DM and T1DM-MVC subjects was measured by ELISA. The horizontal line represents the geometric mean. The p-values were calculated by Kruskal–Wallis one-way analysis of variance. p-value < 0.05 was considered significant. *<0.05; **<0.01 and ***<0.001.

p < 0.001] and those without MVC [8.8(3.8–99.1) pg/ml] when compared with NGT [4.5(3.4–28.7) pg/ml] (Fig. 1d). Serum IL-10 cytokine level was significantly elevated only in T1DM subjects with MVC [14.9(13.0–44.1) pg/ml; p < 0.05], when compared with T1DM without MVC [14.7(13.3–24.2) pg/ml] and NGT [14.8(13.3–24.2) pg/ml] (Fig. 1e). Serum IL-17 levels were significantly elevated in T1DM subjects with MVC [0.65(0.32–3.6) ng/ml; p < 0.001] and those without MVC [0.719(0.03–9.9) ng/ml; P < 0.05] when compared with NGT [0.17 (0.02–9.9) ng/ml] (Fig. 1f). Serum IL-9 cytokine level was significantly elevated both in T1DM subjects with MVC [3.12(2.7–7.3) pg/ml; p < 0.001], and those without MVC [3.2(2.7–4.1) pg/ml; p < 0.001] compared to NGT [2.8(2.6–3.6) pg/ml] (Fig. 1g).

The major findings of the present study are: (1) All the serum cytokines (except for IFN-γ) tested were significantly elevated in T1DM subjects both with and without MVC indicating pan T cell activation and (2) No significant change in the levels of cytokines was noted between those with and without MVC. We found that both the instructor (IL-12) and effector (IFN-γ) cytokines of Th-1 response were elevated in T1DM subjects without MVC. In a longitudinal study conducted by Avanzini et al., 2005, significantly lower percentage of IFN-γ producing CD4+ and CD8+ T cells was seen in T1DM subjects and their high risk relatives compared to healthy controls [9]. With respect to Th2 response, again both the instructor (IL-33) and effector (IL-4) cytokines were significantly elevated in T1DM subjects with and without MVC. IL-4 is the signature cytokine of Th2 cells and is known to trigger autoantibodies against pancreatic autoantigens [10]. Our results are in agreement with a previous report, which showed a sustained elevation of plasma IL-4 levels in pediatric T1DM subjects both before and after the commencement of insulin therapy [11]. Since IL-33 is a novel cytokine, data describing its role in diabetes is limited.

Recently, IL-17 has emerged as a major player in several autoimmune diseases including T1DM, rheumatoid arthritis and multiple sclerosis [5]. Previously, we have shown reduced levels of serum IL-17 in T2DM subjects which were further reduced in those with complications [12]. In the present study, however we found elevated levels of serum IL-17 in T1DM subjects irrespective of MVC. The secretion of IL-9, previously identified as a Th2 cytokine, was recently attributed to a novel CD4+ subset termed Th9 [5]. In the present study, IL-9 levels were significantly elevated in

T1DM subjects both with and without MVC. Our results are in agreement with a recent study which reported an increased frequency of Th9 cells and increased levels of serum IL-9 in T1DM subjects [13]. IL-10 in a remission study conducted by Alizadeh et al. [14] showed no difference between the patient and control groups which is in contrast with what was seen in the current study. The exact reason for this apparent disparity could be due to ethnic differences between the populations studied.

4. Conclusion

The present study suggests a "pan T cell activation" like response in T1DM subjects with all tested cytokines being significantly elevated in T1DM subjects. This cytokine profile was in striking contrast to what was seen in T2DM subjects as reported previously [15]. Interestingly, while T2DM subjects showed classical mixed Th1-Th2 response [15] with suppressed Th17 and Th9 cytokines [12], such a phenomenon was not seen in T1DM subjects. Further, while T2DM with complications showed a pronounced Th-1 polarization such a phenomenon was not seen in T1DM subjects with MVC. The exact immunological basis for these apparent differences in the serum cytokine profiles between T1DM and T2DM is not known and warrants further investigations. Since the aim of this study was to draw a serum cytokine profile, characterizing the Th cells which secrete these cytokines was beyond the scope of this report. The major limitation of our study is its cross-sectional nature and hence no cause and effect relationship can be ascertained. However, the major strength of this study is that it is the first study on all the major adaptive immune cytokines in T1DM subjects (both with and without MVC), in an Asian Indian population.

Conflict of interest

None.

Author's contribution

VA, AA, and VM conceived and designed the experiments. SS performed the experiments. VA, AA and VM analyzed the data and SS wrote the paper.

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