



## Peripheral blood mononuclear cells of patients with latent autoimmune diabetes secrete higher levels of pro- & anti-inflammatory cytokines compared to those with type-1 diabetes mellitus following *in vitro* stimulation with $\beta$ -cell autoantigens

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**Background & objectives:** Type-1 diabetes mellitus (T1DM) and latent autoimmune diabetes in adults (LADA) share similar pathological features but differ in age of onset and progression. There is a scarcity of information on differences in CD4<sup>+</sup> T-cell responses, particularly, cytokine secretion, between the two forms of autoimmune diabetes. Here proliferative potential and concentration of pro- and anti-inflammatory cytokines secreted by peripheral blood mononuclear cells (PBMCs) of T1DM and LADA patients were compared, after *in vitro* stimulation with  $\beta$ -cell autoantigens.

**Methods:** A total of 19 patients with LADA, 37 with T1DM and 20 healthy controls were compared on the basis of lymphocyte proliferation and secretion of pro- and anti-inflammatory cytokines belonging to different T-helper types after *in vitro* stimulation of PBMCs with insulin and glutamic acid decarboxylase 65 (GAD65).

**Results:** Following insulin stimulation, LADA group secreted higher concentration of interleukin-17 (IL-17) ( $P=0.02$ ) and had higher proportion of interferon gamma (IFN- $\gamma$ ) secretors ( $P<0.001$ ) than T1DM group. Post-GAD65 stimulation, higher proportion of LADA patients secreted IL-23 than T1DM group ( $P=0.02$ ). Proportion of responders, as well as levels of secreted IL-10, were significantly higher in LADA than T1DM group, following stimulation with both insulin ( $P=0.01$ ) and GAD65 ( $P=0.03$ ). A significant positive correlation was observed between body mass index and IL-17 levels ( $r=0.41$ ,  $P=0.04$ ) and fasting plasma C-peptide with IL-10 levels ( $r=0.37$ ,  $P=0.04$ ).

**Interpretation & conclusions:** There are differences in the portfolio of cytokine secretion in diabetic subjects with varying rates of  $\beta$ -cell destruction as LADA subjects secrete higher levels of both pro- and anti-inflammatory cytokines on exposure to  $\beta$ -cell autoantigens, thus highlighting another distinguishing feature in the pathophysiology of the two forms of autoimmune diabetes.

**Key words** CD4<sup>+</sup> T-cells - cytokines - glutamic acid decarboxylase 65 - insulin - latent autoimmune diabetes in adults - type-1 diabetes mellitus

Type-1 diabetes mellitus (T1DM) is an organ-specific autoimmune disease caused by selective destruction of insulin-producing  $\beta$ -cells in the pancreatic islets mainly by the autoreactive T-cells. A slower progressive form of this disease latent autoimmune diabetes in adults (LADA) has similar pathological features and accounts for 10 per cent of total diabetes cases but usually misdiagnosed as type-2 diabetes mellitus (T2DM)<sup>1,2</sup>. Insulin and glutamic acid decarboxylase 65 (GAD65) play an important role in disease pathogenesis as these are the earliest autoantigens recognized by autoreactive CD4+ T-cells<sup>3,4</sup>. Studies from India have also reported prevalence of islet-associated antibodies in patients with varying durations of T1DM<sup>5,6</sup>.

Cytokines are important inflammatory mediators and are implicated in the pathogenesis of autoimmune diabetes<sup>7</sup>. While the role of interferon gamma (IFN- $\gamma$ ) has been well documented as a major proinflammatory cytokine<sup>8</sup>, the role of interleukin-17 (IL-17) is increasingly being recognized in the pathogenesis of autoimmune diabetes<sup>9,10</sup>. It is suggested that T-helper 17 (Th17) cells may be actively involved in early stages of T1DM as higher levels of IL-17 coupled with increased IL-17 gene expression were detected in new-onset patients as compared to long-standing ones<sup>9,11</sup>. IL-6 has also been implicated in pathogenesis of both T1DM and T2DM as an important proinflammatory mediator<sup>12</sup>. To suppress autoreactive T-cell responses, regulatory T-cells (Tregs) produce anti-inflammatory cytokines, IL-10 and transforming growth factor beta<sup>13</sup>. In T1DM patients, Tregs are functionally defective with markedly reduced suppressive activity<sup>14,15</sup>. Another Th2 cytokine IL-4 counter-regulates the effects of IFN- $\gamma$ <sup>16</sup>.

In humans, it is difficult to study local cytokine milieu during disease progression due to inaccessibility of pancreas. LADA represents a unique subset of autoimmune diabetes with slow but ongoing  $\beta$ -cell destruction, and these individuals can provide valuable information on CD4+ T-cell function during disease progression. We observed decreased proliferative potential and pathogenicity of preproinsulin-specific CD8+ T-cells in individuals with LADA as compared to T1DM patients, suggestive of lower intensity of CD4+ T-cell pro-inflammatory responses in these individuals<sup>17</sup>. There are a few reports on comparison of cytokines in peripheral circulation in patients with T1DM and LADA, but they did not observe any distinction between the two groups<sup>18-20</sup>. One such study compared secretion of IFN- $\gamma$  and IL-13 in patients with

T1DM and LADA following stimulation with  $\beta$ -cell-associated peptides, and demonstrated no significant differences between the two groups in terms of number of cytokine-producing cells<sup>20</sup>. The present work was aimed to study differences in patients with T1DM and LADA in the proliferative potential of lymphocytes and secretion of cytokines belonging to different Th types, including IFN- $\gamma$ , IL-17, IL-23, IL-6, IL-10 and IL-4 following *in vitro* stimulation of their peripheral blood mononuclear cells (PBMCs) with major  $\beta$ -cell autoantigens, insulin and GAD65.

### Material & Methods

The study was conducted in the department of Endocrinology at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, from May 2012 to April 2015. A total of 76 individuals, consisting of 37 recently diagnosed T1DM patients and 19 with LADA, were selected from the departments of Paediatrics and Endocrinology, PGIMER, along with 20 age- and sex-matched non-diabetic healthy controls (HCs), majority of whom were siblings of T1DM and LADA patients. Diabetes was diagnosed as per the American Diabetes Association criteria<sup>21</sup>. Inclusion criteria for autoimmune diabetes were the presence of autoantibodies to GAD65 or islet antigen-2 or islet cell antigen or insulin. Inclusion criteria for LADA were age >25 yr, positive for at least one of the autoantibodies and insulin independence for at least six months after diagnosis of diabetes; in addition, none of the LADA patients had received exogenous insulin therapy at the time of recruitment. Exclusion criteria included anaemia (Hb <8.0 g/dl), any acute illness, other autoimmune diseases (including celiac disease), lymphomas, psychiatric illness and pregnancy.

The study protocol was approved by the Institutional Ethics Committee. After obtaining informed consent in writing, fasting peripheral blood samples were obtained from all participants in heparinized vacutainers. In addition to screening for autoantibodies, titres of anti-insulin (Orgentec Diagnostika, Mainz, Germany) and anti-GAD65 (Aesku Diagnostics, Wendelsheim, Germany) antibodies were analyzed by ELISA. The minimum detectable level (with intra-, inter-assay CV) for anti-insulin was 0.5 U/ml (3.1, 5.1%) and anti-GAD65 was 6 IU/ml (7.9, 7.3%). Fasting plasma C-peptide levels were determined by electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany), while glycated haemoglobin (HbA<sub>1c</sub>) was

determined by spectrophotometry following cation-exchange chromatography (Bio-Rad, Hercules, USA).

**Peripheral blood mononuclear cells (PBMCs) stimulation and proliferation:** PBMCs were isolated by density-gradient centrifugation with Ficoll. For stimulation,  $10^6$  PBMCs per well were cultured in duplicate separately with optimized concentrations of insulin (10.0  $\mu\text{g/ml}$ ) and GAD65 (5.0  $\mu\text{g/ml}$ ) (Sigma-Aldrich, USA) in RPMI-1640 medium, supplemented with 0.1 per cent penicillin-streptomycin and 10 per cent foetal calf serum in 24-well tissue culture plates for 72 h at 37°C with 5 per cent  $\text{CO}_2$ <sup>17,22</sup>. After completion of incubation period, culture supernatants were collected by centrifugation and stored at  $-80^\circ\text{C}$  in triplicates to avoid multiple freeze-thaw cycles until cytokine analysis. Lymphocyte proliferation was assessed by flow cytometry using carboxyfluorescein succinimidyl ester (CFSE) dye dilution assay<sup>23</sup>. Briefly,  $2 \times 10^6$  PBMCs were stained with CFSE dye, of which  $2 \times 10^5$  cells were acquired along with unstained cells and  $2 \times 10^5$  cells/well were stimulated in triplicates. After incubation, cells were harvested, acquired and analyzed for proliferation on flow cytometer (FACS Canto II, BD Biosciences, USA). In all experiments, phytohaemagglutinin (PHA) (5.0  $\mu\text{g/ml}$ ) was used as positive control.

**Extracellular cytokine analysis:** ELISA was performed to determine the concentrations of cytokines in culture supernatants including, IFN- $\gamma$ , IL-4 (Diaclone, Cedex, France), IL-10, IL-17 (subunits A-F) (Boster Biological Technology, Pleasanton, USA), IL-23 (Cusabio Biotech, Wuhan, China) and IL-6 (Mabtech, Nacka Strand, Sweden) as per manufacturer's instructions. The minimum detectable level (with intra-, inter-assay CV) for IL-4 was 0.31 pg/ml (9.2, 13.7%), IFN- $\gamma$ , 0.69 pg/ml (3.9, 8.6%), IL-23, 6.25 pg/ml (8, 10%), IL-17, 1.0 pg/ml (5.9, 8.4%), IL-10, 0.5 pg/ml (5.7, 7.4%) and IL-6, 1.0 pg/ml (2.5, 3.8%), respectively. In case of GAD65 stimulation, culture supernatants of only T1DM and LADA groups were analyzed. IL-6 secretion was analyzed only in case of *in vitro* stimulation with insulin. Responders were defined as those secreting detectable amount of a cytokine at the end of the incubation period, as compared to baseline secretion at zero hour. Responders were compared between T1DM and LADA groups, as well as between HC and diabetic (T1DM+LADA) groups.

**Statistical analysis:** For comparison, the diabetic group comprised both T1DM and LADA patients

taken together and results were presented as mean $\pm$ standard error of mean (SEM). Unpaired *t* test was used to compare means between different groups, while Mann–Whitney U test was used when data were not normally distributed. Fisher's exact test was used to analyze differences in the proportions of patients secreting detectable amount of cytokines. Bivariate correlations between concentrations of individual cytokines with body mass index (BMI), fasting plasma C-peptide levels, anti-insulin and anti-GAD65 antibody titres were analyzed by Spearman's correlation test. Multivariate regression analysis was performed to investigate differences of cytokine concentrations (dependent variables) between different participant groups adjusted for BMI, age and C-peptide (independent variables). The data were log transformed to normalize the values of cytokines. Five models were used, which were adjusted for different combinations of variables: model 1, age; model 2, age and BMI; model 3, age, BMI and C-peptide; model 4, C-peptide; model 5, age and C-peptide; model-6, HbA<sub>1c</sub>. All the statistical analyses were performed using Graphpad Prism (v-4.0, La Jolla, CA, USA), SPSS (version 21.0, Armonk, NY, USA) and Microsoft Office Excel (2007).

## Results

The mean ( $\pm$ SEM) age of LADA group individuals (28.8 $\pm$ 1.6 yr) was significantly higher than T1DM patients (7.9 $\pm$ 0.5 yr) ( $P < 0.01$ ) at the time of enrolment. Mean fasting plasma C-peptide levels were significantly higher in LADA (1.09 $\pm$ 0.19 ng/ml) than T1DM patients (0.48 $\pm$ 0.08 ng/ml) ( $P < 0.01$ ), whereas mean HbA<sub>1c</sub> (%), (mmol/mol) was significantly higher in T1DM (11.65 $\pm$ 0.44, 103 $\pm$ 2.5) versus those with LADA (9.56 $\pm$ 0.42, 81 $\pm$ 2.2) ( $P < 0.01$ ). None of the participants had a history of any other autoimmune disease including celiac disease. The BMI of T1DM patients (16.34 $\pm$ 0.69 kg/m<sup>2</sup>) was significantly lower than those with LADA (20.27 $\pm$ 0.74 kg/m<sup>2</sup>) ( $P < 0.01$ ). Among those with diabetes, percentage of patients with T1DM and LADA demonstrating anti-insulin and anti-GAD65 antibodies had no significant difference (Table I). On comparison of antibody titres, no significant difference was observed between both the groups (data not shown).

**Lymphocytes proliferation following exposure to  $\beta$ -cell autoantigens:** Following *in vitro* stimulation with insulin and GAD65, proliferation of lymphocytes was observed in all individuals (Fig. 1)

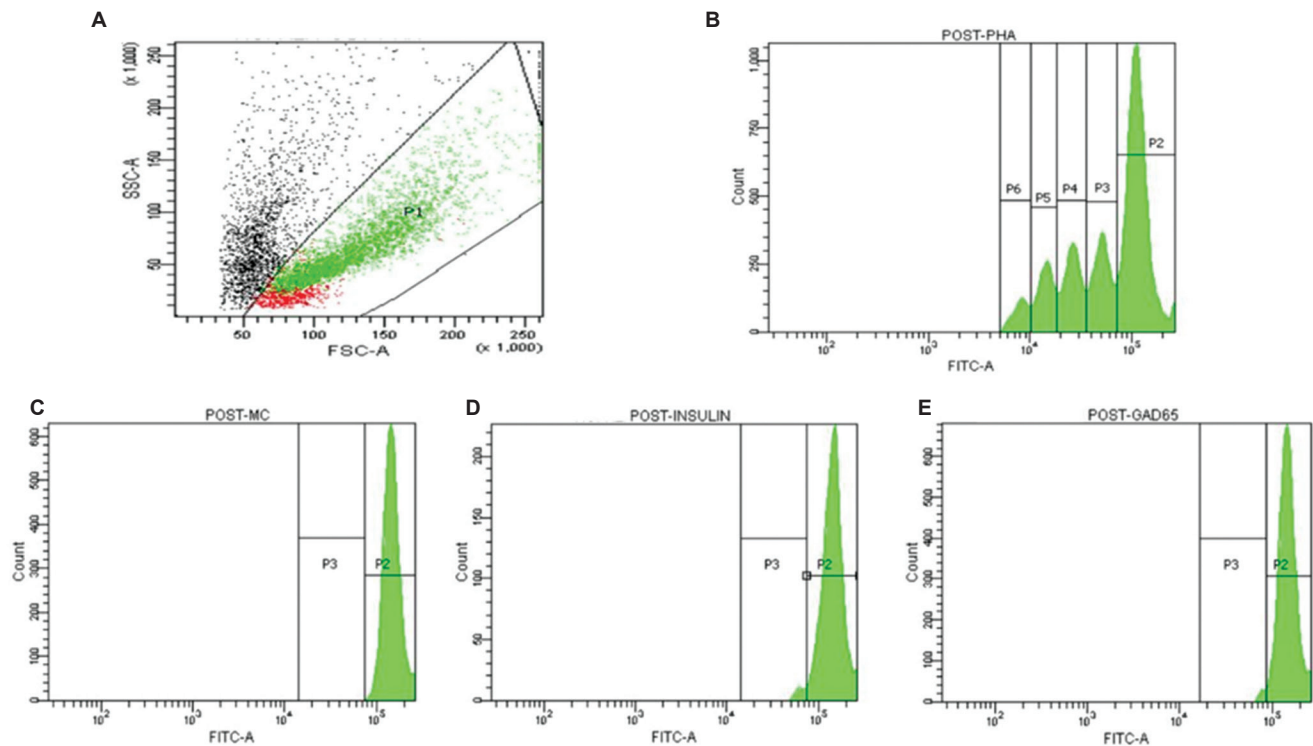
**Table I.** Clinical characteristics of patients and healthy controls and proliferation indices of their lymphocytes following *in vitro* stimulation with insulin, GAD65 and PHA

Characteristic	T1DM (n=37)	LADA (n=19)	HC (n=20)
Age (yr)	7.9±0.5	28.8±1.6**	17.4±2.0
Sex (M/F)	20/17	11/8	10/10
Fasting plasma C-peptide (ng/ml)	0.48±0.08	1.09±0.19**	2.96±0.61
HbA <sub>1c</sub> (%; mmol/mol)	11.65±0.44, 103±2.5	9.56±0.42**, 81±2.2**	5.31±0.09, 35±0.75
BMI (kg/m <sup>2</sup> )	16.34±0.69	20.27±0.74**	23.2±1.42
Anti-insulin antibodies (% individuals)	16/37 (43%)	9/19 (47%)	-
Anti-GAD65 antibodies (% individuals)	25/37 (68%)	8/19 (42%)	1/20 (5%)
Insulin (10.0 µg/ml) (Proliferation index)	1.14±0.01	1.02±0.02	1.01±0.03
GAD65 (5.0 µg/ml) (Proliferation index)	1.03±0.02	1.01±0.03	0.96±0.02
PHA (5.0 µg/ml) (Proliferation index)	3.20±0.22	2.99±0.18	2.97±0.22

M/F, Male/female; HbA<sub>1c</sub>, Glycated haemoglobin; BMI, Body mass index; GAD, Glutamic acid decarboxylase;

PHA, Phytohaemagglutinin; T1DM, Type 1 diabetes mellitus; LADA, Latent autoimmune diabetes in adults; HC, Healthy controls.

\*\**P*<0.01 compared to T1DM, No significant difference was observed in the mean proliferation index of lymphocytes between any of the groups following *in vitro* stimulation with insulin or GAD65

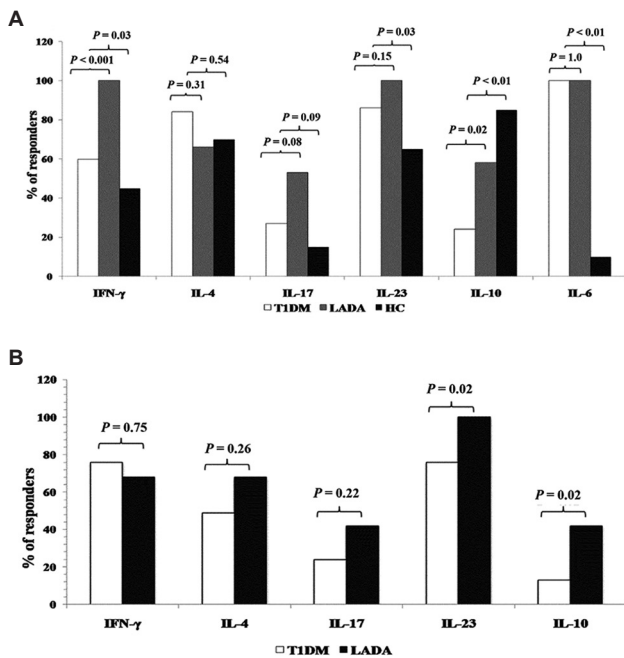


**Fig. 1.** Representative images showing proliferation of lymphocytes stained with carboxyfluorescein succinimidyl ester (CFSE) following *in vitro* stimulation with autoantigens, insulin (10.0 µg/ml) and glutamic acid decarboxylase 65 (GAD65, 5.0 µg/ml), positive control, phytohaemagglutinin (PHA, 5.0 µg/ml) and negative control, media control (MC). (A) Lymphocytes were gated according to forward and side scatter. Histograms represent cells stimulated with (B) PHA, (C) media control, (D) insulin, (E) GAD65.

and the mean proliferation index of lymphocytes was similar in patients with T1DM, LADA and HCs (Table I).

**Cytokine analysis:** Cytokine responses were clustered into four groups based on the type of cytokines secreted, as proinflammatory *i.e.* Th1 (IFN- $\gamma$ ) and





**Fig. 2.** Percentage of individuals secreting various cytokines following *in vitro* stimulation of peripheral blood mononuclear cells. (A) Post-insulin stimulation, latent autoimmune diabetes in adults (LADA) group showed significantly higher number of responders secreting interferon gamma (IFN- $\gamma$ ) and interleukin-10 (IL-10). (B) Post-glutamic acid decarboxylase 65 (GAD-65) stimulation, LADA group showed significantly higher responders secreting IL-23 and IL-10. Interleukin-6 secretion was compared only in case of insulin stimulation. Responders were defined as those secreting detectable cytokine levels at the end of incubation period, as compared to zero hour.

Th17 (IL-17 and IL-23) and anti-inflammatory *i.e.* Treg (IL-10) and Th2 (IL-4, IL-6). In unstimulated cultures, the mean concentration of all cytokines, except IL-10 (3.6 $\pm$ 0.25 pg/ml), was negligible compared to the insulin and GAD65 stimulated cultures.

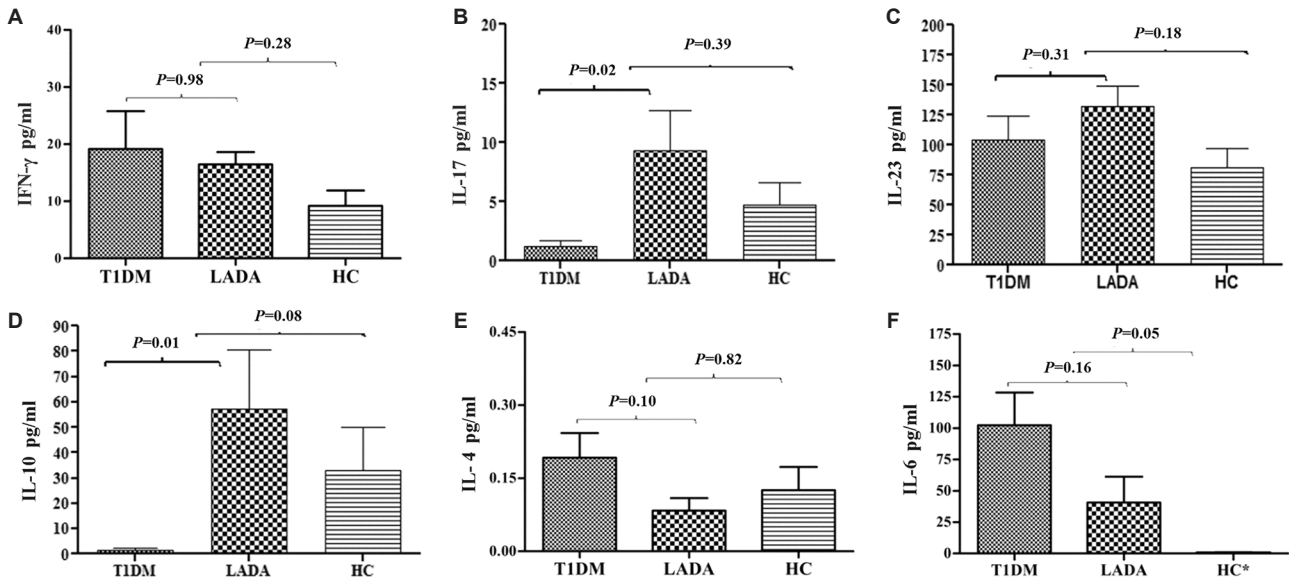
**IFN- $\gamma$  secretion in patients with LADA following insulin stimulation:** After *in vitro* stimulation with insulin, IFN- $\gamma$  secretion was observed from PBMCs of all of the 19 (100%) LADA patients, significantly higher than those with T1DM (22/37, 59.5%) ( $P$ <0.001) and HC subjects (9/20, 45%) ( $P$ =0.03) (Fig. 2A). However, comparison of mean concentration of secreted IFN- $\gamma$  did not yield any significant difference between the T1DM (19.94 $\pm$ 6.19 pg/ml) and LADA group (16.28 $\pm$ 3.4 pg/ml). No significant difference was observed between the diabetic (18.35 $\pm$ 3.74 pg/ml) and HC groups (9.20 $\pm$ 2.70 pg/ml) (Fig. 3A) in terms of IFN- $\gamma$  secretion. In response to *in vitro* stimulation with GAD65, the number of responders was similar in both T1DM (28/37, 76%) and LADA (13/19, 68%) groups

(Fig. 2B). Likewise, both T1DM (9.89 $\pm$ 3.43 pg/ml) and LADA patients (9.32 $\pm$ 3.16 pg/ml) demonstrated similar levels of IFN- $\gamma$  (Fig. 4A).

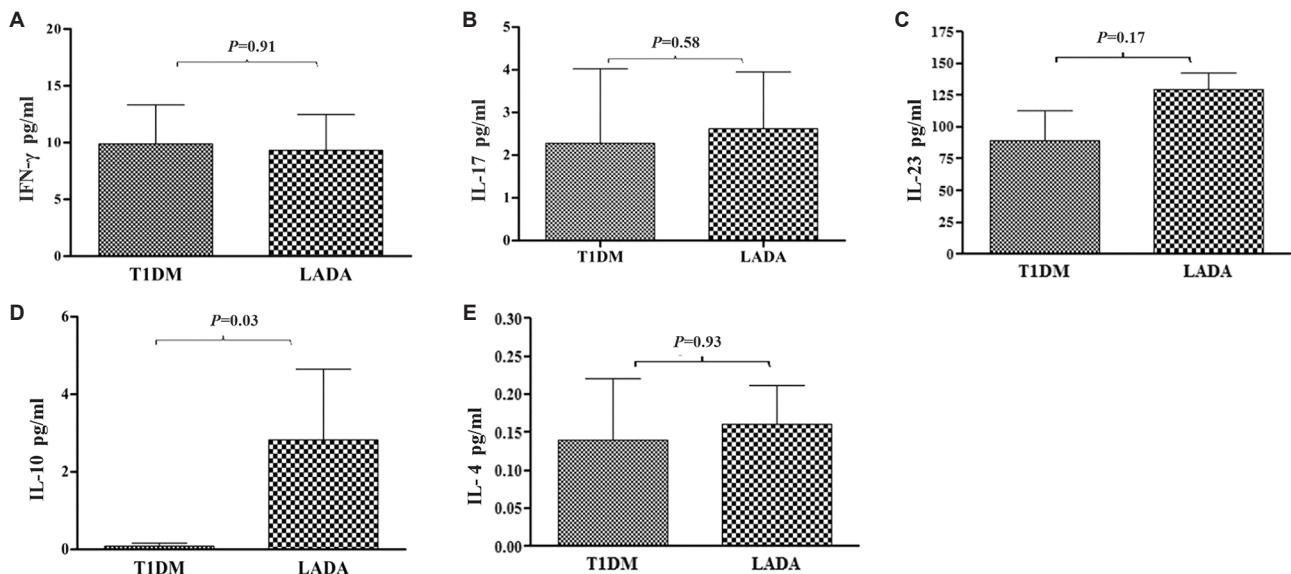
**T-helper 17 (Th17) responses in LADA patients following stimulation with insulin and GAD65:** To determine Th17 responses following *in vitro* stimulation of PBMCs with insulin and GAD65, concentrations of IL-17 and IL-23 were assessed. After stimulation with insulin, the number of patients secreting IL-17 was significantly higher in LADA (10/19, 53%) than T1DM groups (10/37, 27%) ( $P$ =0.08) (Fig. 2A) and mean concentration of secreted IL-17 was significantly higher in LADA (9.29 $\pm$ 3.43 pg/ml) than T1DM group (1.18 $\pm$ 0.56 pg/ml) ( $P$ =0.02) (Fig. 3B). Compared to the HC group (3/20, 15%), the proportion of responders were expectedly higher in diabetic group following insulin stimulation ( $P$ =0.09) (Fig. 2A). Following GAD65 stimulation, secretion of IL-17 was observed in only 9/37 (24%) T1DM and 8/19 (42%) LADA patients ( $P$ =0.22) (Fig. 2B), and the mean ( $\pm$ SEM) concentration of IL-17 was similar in both LADA (2.62 $\pm$ 1.32 pg/ml) and T1DM subjects (2.29 $\pm$ 1.75 pg/ml) (Fig. 4B).

IL-23 secretion by PBMCs in response to *in vitro* insulin stimulation was detected in most individuals, with all of the LADA, 32/37 (86%) T1DM patients and 13/20 HC (65%) demonstrating detectable IL-23 in culture supernatants (Fig. 2A). IL-23 secretion by PBMCs was similar in LADA (132.4 $\pm$ 17.10pg/ml) and T1DM patients (103.8 $\pm$ 20.47pg/ml) and no difference was observed between the diabetic (116.2 $\pm$ 13.80 pg/ml) and HC group (81.01 $\pm$ 15.82 pg/ml) (Fig. 3C). Following *in vitro* stimulation with GAD65, all of the LADA patients secreted detectable IL-23 which was significantly higher than T1DM patients (28/37, 76%) ( $P$ =0.02) (Fig. 2B). However, both the groups demonstrated similar levels of secreted IL-23 [T1DM (89.20 $\pm$ 23.79 pg/ml) and LADA (129.9 $\pm$ 13.01 pg/ml)] (Fig. 4C).

**IL-10 secretion in LADA patients following stimulation with insulin and GAD65:** In response to *in vitro* stimulation of PBMCs with insulin, 11 of 19 (58%) LADA patients secreted IL-10, which was significantly higher than those with T1DM (9/37, 24%) ( $P$ =0.02). Compared to diabetic group, the IL-10 producers were significantly higher in the HC group (17/20, 85%) ( $P$ <0.01) (Fig. 2A). On comparison of mean concentration



**Fig. 3.** Extracellular levels of cytokines, (A) IFN- $\gamma$ , (B) IL-17, (C) IL-23, (D) IL-10, (E) IL-4 and (F) IL-6 in culture supernatants of peripheral blood mononuclear cell (PBMC) cultures in type-1 diabetes mellitus (T1DM), LADA and healthy control (HC) groups after *in vitro* stimulation with insulin. Values are shown as mean $\pm$ standard error of mean. Levels of secreted interleukin-17 ( $P=0.02$ ) and interleukin-10 ( $P=0.01$ ) were significantly higher in LADA group than T1DM group post insulin stimulation. PBMCs of HC group secreted very low levels of IL-6 that could not be observed on the depicted scale.



**Fig. 4.** Extracellular levels of cytokines, (A) IFN- $\gamma$ , (B) IL-17, (C) IL-23, (D) IL-10 and (E) IL-4 in culture supernatants of peripheral blood mononuclear cells (PBMCs) isolated from T1DM and LADA group individuals after *in vitro* stimulation with GAD-65. Values are shown as mean $\pm$ standard error of mean.

significantly higher levels of IL-10 were observed in LADA ( $57.12\pm 23.70$  pg/ml) than in T1DM group ( $1.48\pm 1.05$  pg/ml) ( $P=0.01$ ) (Fig. 3D). Overall, the production of IL-10 by PBMCs obtained from the diabetic group ( $25.33\pm 11.60$  pg/ml) was significantly lower than the HC group ( $32.91\pm 17.01$  pg/ml) ( $P=0.08$ ). Like in case of insulin, stimulation with

GAD65 yielded similar results with significantly higher proportion of LADA patients (8/19, 42%) secreting detectable IL-10 compared to T1DM patients (5/37, 13%) ( $P=0.02$ ) (Fig. 2B), with LADA group ( $2.82\pm 1.82$  pg/ml) demonstrating higher mean concentration of secreted IL-10 than T1DM group ( $0.08\pm 0.08$  pg/ml) ( $P=0.03$ ) (Fig. 4D).

**IL-4 levels in T1DM & LADA patients following insulin and GAD65 stimulation:** Following *in vitro* stimulation with insulin, PBMCs of most of the individuals secreted very low levels of IL-4. The number of responders was similar in T1DM (31/37, 84%) and LADA groups (13/19, 68%) (Fig. 2A), and no significant difference was observed in mean concentrations of IL-4 in both the groups [T1DM (0.19±0.06 pg/ml) and LADA (0.08±0.03 pg/ml)] (Fig. 3E). The diabetic and the HC groups also did not demonstrate any differences in the number of responders (14/20, 70%) and levels of secreted IL-4 (0.13±0.04 pg/ml vs. 0.13±0.05 pg/ml). Response to GAD65 stimulation was also analogous, with number of patients secreting detectable IL-4 being comparable in T1DM (18/37, 49%) and LADA group (13/19, 68%) (Fig. 2B), with no significant difference in levels of secreted IL-4 [T1DM (0.14±0.08 pg/ml) and LADA (0.16±0.05 pg/ml)] (Fig. 4E).

**IL-6 secretion in T1DM & LADA patients following stimulation with insulin:** After *in vitro* stimulation with insulin, IL-6 secretion was observed from PBMCs of all T1DM and LADA patients, significantly higher than the HC (2/20, 10%) ( $P<0.01$ ) (Fig. 2A). On comparing the mean concentration of cytokines, higher levels were observed in T1DM (102.5±27.65 pg/ml) than LADA group (40.88±23.12 pg/ml) though the difference was not significant. The HCs (0.35±0.09 pg/ml) secreted very low levels of IL-6 than the diabetic group (75.11±19.11 pg/ml ( $P=0.05$ )) (Fig. 3F).

**Positive association between IL-17 with BMI and IL-10 with C-peptide in autoimmune diabetes:** All T1DM and LADA patients were pooled in one group, and correlation analysis was performed between concentration of individual cytokines secreted by PBMCs stimulated *in vitro* with insulin and BMI. Of all the cytokines, only IL-17 levels showed a significant positive correlation

with BMI ( $r=0.41$ ,  $P=0.04$ ) (Table II). To observe the effect of remnant  $\beta$ -cell mass and hyperglycaemia on cytokine secretion pattern of PBMCs, fasting plasma C-peptide levels were correlated with HbA<sub>1c</sub> separately with concentrations of individual cytokines. A significant positive correlation was observed between

**Table II.** Correlation between levels of cytokines secreted by insulin stimulated peripheral blood mononuclear cells (PBMCs) with BMI, fasting plasma C-peptide and HbA<sub>1c</sub> in patients with autoimmune diabetes

Correlated parameters	<i>r</i>	<i>P</i>
<b>BMI and cytokines</b>		
IFN- $\gamma$	-0.04	0.85
IL-4	0.20	0.42
IL-17	0.41	0.04
IL-23	0.30	0.13
IL-10	0.14	0.50
IL-6	-0.07	0.80
<b>C-peptide and cytokines</b>		
IFN- $\gamma$	-0.17	0.37
IL-4	-0.02	0.94
IL-17	0.17	0.34
IL-23	0.06	0.74
IL-10	0.37	0.04
IL-6	-0.03	0.91
<b>HbA<sub>1c</sub> and cytokines</b>		
IFN- $\gamma$	0.15	0.40
IL-4	-0.18	0.42
IL-17	-0.09	0.63
IL-23	0.11	0.52
IL-10	-0.12	0.51
IL-6	-0.20	0.42
HbA <sub>1c</sub> , Glycated haemoglobin; BMI, Body mass index; IFN- $\gamma$ , Interferon gamma; IL, Interleukin		

**Table III.** Comparisons between LADA and T1DM groups following adjustment of confounding variables

Cytokine	Model-1	Model-2	Model-3	Model-4	Model-5	Model-6
IL-17	1.083**	1.041**	1.003*	0.182	1.020**	-0.258*
IL-4	-1.242**	-1.206*	-1.164**	-0.219	-1.170**	-0.596***
IL-10	0.356	0.411*	0.390	0.610***	0.331	-0.299*
IL-23	0.119	0.119	0.129	0.275	0.136	0.200
IFN- $\gamma$	0.263	0.285	0.291	0.413*	0.275	-0.039
IL, interleukin; IFN- $\gamma$ , interferon gamma; T1DM, type 1 diabetes mellitus; LADA, latent autoimmune diabetes in adults. Data is shown in the form of $\beta$ coefficient values obtained following adjustment analysis. Multiple linear regression models were created for IL-17, IL-4, IL-10, IL-23 and IFN- $\gamma$ . Cytokines were entered into the models as log-transformed variables. Model 1, age; model 2, age and BMI; model 3, age, BMI and C-peptide; Model-4, C-peptide; Model-5, age and C-peptide; Model-6, HbA <sub>1c</sub> . $P^*<0.05$ , $^{**}<0.01$ , $^{***}<0.001$						



IL-10 and C-peptide ( $r=0.37$ ,  $P=0.04$ ), whereas no correlation of fasting plasma C-peptide levels was observed with any of the other cytokines; further, no correlation was observed between  $HbA_{1c}$  and any of the cytokine (Table II). Since insulin and GAD65 were used as major stimulants in cytokine analysis, correlation of anti-insulin and anti-GAD65 antibody titres with all cytokines was also assessed separately; however, no correlation was observed between antibody titres and any of the cytokines (data not shown).

*Adjustment of confounding variables for analysis of cytokine data in relation to bivariate comparison of groups:* The influence of potential confounding factors such as age, BMI, C-peptide and  $HbA_{1c}$  was investigated on differences in levels of cytokines between the groups by employing various multiple regression models (Table III).

After stepwise adjustment of confounding factors, the differences in the concentration of IL-17 between the T1DM and LADA patients were still observed. Although the concentration of IL-4 was low in both the groups as observed during bivariate comparison (Fig. 3), the mean levels became significantly lower in LADA group following adjustment analysis. For IL-10, the differences observed between the two groups were similar following stepwise adjustment of most models including  $HbA_{1c}$  alone, except for model 1 and 5; for model 3, the difference was nearly significant (Table III).

### Discussion

The underlying reasons for the slow progression of disease in LADA are not well understood. As indicated by plasma C-peptide levels, most of the LADA patients included in our study had appreciable residual  $\beta$ -cell mass at the time of diagnosis, suggestive of slower disease progression as compared to T1DM where  $\beta$ -cell mass was almost destroyed. This study was done to investigate cytokine profile of different Th types including proinflammatory (Th1, Th17) and anti-inflammatory (Treg, Th2) cytokines secreted by peripheral lymphocytes on exposure to  $\beta$ -cell autoantigens not only to understand differences if any, with the T1DM patients, but also to assess cytokines associated with disease progression, as patients with LADA represent those with ongoing pancreatic inflammation.

In our study it was possible to distinguish between T1DM and LADA patients on the basis of responders

and concentration of secreted cytokines. This was in contrast to the previous reports advocating that T1DM and LADA are immunologically similar and cannot be differentiated on the basis of cytokine levels<sup>18,19</sup>. The LADA group in our study secreted higher levels of IL-17 after stimulation with insulin as compared to T1DM group. All patients were recently diagnosed, and hence, the duration of insulin therapy in T1DM patients was too less to have any impact on cytokine secretion following *in vitro* insulin stimulation. Similarly, more LADA patients secreted IFN- $\gamma$  post-insulin stimulation and IL-23 post-GAD65 stimulation. In another previous ELISpot-based study<sup>20</sup>, IFN- $\gamma$  secreting cells after GAD65 stimulation were similar in T1DM and LADA groups. Such discrepancies observed might be explained by differences in assay types, duration and concentration of stimuli and ethnicity of individuals. Increased secretion of proinflammatory cytokines by LADA patients could be attributed to the ongoing inflammation of  $\beta$ -cells, providing abundant antigenic target for the activated CD4+ T-cells, compared to T1DM patients, although it remains to be seen whether Th1 or Th17 responses prevail with the same intensity eventually when LADA patients lose  $\beta$ -cell mass and become insulin dependent. This finding was supported by the evidence of lower IL-4 production by the LADA group, following adjustment analysis, indicative of a poor Th2 response to counter the IFN- $\gamma$ -mediated Th1 response. However, the IL-10 responses were higher in LADA group in terms of number of secretors and concentration, indicating that the immunosuppressive arm of the  $\beta$ -cell-specific Tregs was active and perhaps contributed to the slow rate of  $\beta$ -cell destruction. This was further confirmed when a positive correlation was observed between fasting plasma C-peptide levels and secreted IL-10 levels. It is known that IL-10 carries out immune regulation mainly by suppressing the release of inflammatory cytokines from other cell types, besides blocking B7/CD28 costimulatory pathway, inhibiting dendritic cell maturation and reducing major histocompatibility complex (MHC) class II expression<sup>24,25</sup>. Given the fact that  $\beta$ -cells die by multiple pathways, it must be noted that local cytokine milieu in the pancreas is a cumulative outcome of pro- and anti-inflammatory cytokines secreted by  $\beta$ -cell-specific CD4+ T-cells. Even though we did not investigate pancreatic IL-10 levels, it could be possible that there is local preponderance of IL-10 secreting CD4+ T-cells in the pancreatic region which is reflected in their measured peripheral T-cell responses. In addition, the concentration of IL-10 secreted by



PBMCs of HCs was higher than diabetic patients, underlining the importance of functional Tregs<sup>14,15</sup> and IL-10 in suppression of autoimmune diabetes<sup>24</sup>.

Most of our T1DM patients having negligible C-peptide levels demonstrated production of proinflammatory cytokines IL-17, IFN- $\gamma$ , IL-6 and IL-23 while the levels of IL-10 and IL-4 were very low, indicating the dominance of proinflammatory over anti-inflammatory responses in T1DM. It confirms that memory CD4<sup>+</sup> T-cells are still active in these patients even after absolute obliteration of  $\beta$ -cell mass and can mount a heightened pro-inflammatory response following re-exposure to  $\beta$ -cell autoantigens<sup>22,25</sup>. It has been suggested that initially there is inclination of autoreactive CD4<sup>+</sup> T-cells towards a Th17 phenotype, which later transforms to Th1 type<sup>9</sup>. In this study, a significant difference was observed in the levels of secreted IL-17 between T1DM and LADA patients. Therefore, the role of local antigen presenting cells in priming  $\beta$ -cell-specific CD4<sup>+</sup> T-cells towards a particular proinflammatory type may be relevant. Further, analysis of immunophenotype of antigen-specific CD4<sup>+</sup> T-cells at initial stages of disease progression could provide more information about the cytokines involved and the environmental cues that favour a particular Th signature in pathogenesis of autoimmune diabetes. Differences in CD4<sup>+</sup> T-cell immunophenotype are expected as observed in case of antigen-specific CD8<sup>+</sup> T-cells reported by our group<sup>17</sup>.

Association between concentration of proinflammatory cytokines and obesity has been shown<sup>26</sup>. In our patients with autoimmune diabetes, particularly LADA, we observed a positive correlation between BMI and IL-17 indicating that IL-17 might be part of proinflammatory cytokines leading to high risk of diabetes, including type-2 diabetes, where its role is suggested by several studies<sup>19,27</sup>. Increased Th17 cells have been observed in obese individuals causing metabolic dysfunction in muscle and liver cells *in vitro*<sup>28</sup>; in addition, high IL-17 levels were observed in obese than lean individuals<sup>29</sup>. Although most of the LADA patients in our study did not have obese phenotype, their BMI was significantly higher than T1DM patients. Therefore, our data merely indicate but not enough to provide evidence that increased activity of IL-17 secreting CD4<sup>+</sup> T-cells is influenced by BMI and contributes to disease progression in patients with LADA.

There were some limitations in this study, such as the sample size; however, it must be considered that

LADA patients were difficult to find. The minimum age of LADA patients included was chosen as 25 yr so as to include more individuals with late-onset diabetes and compared them with significantly younger individuals with early onset *i.e.* T1DM. Most of the patients in our study were either pre-pubertal (T1DM) or adults (LADA); therefore, any puberty-associated variations in immune responses were automatically eliminated. It must also be noted that the cytokine secretion profile of patients was compared following stimulation with  $\beta$ -cell autoantigens and not just cytokines in circulation. The study design could be further improved by conducting a longitudinal follow up study involving stimulation of PBMCs obtained at different time points to get a better insight of cytokine secretion pattern at different stages of progression of autoimmune diabetes. In addition, the phenotype of T-cells, including activated/memory/regulatory subsets, co-expression of cytokines can be assessed to have a better picture of T-cell responses involved in disease progression. In this context, the results obtained from stimulation assays in this study are preliminary.

In conclusion, our findings demonstrated that patients with LADA could be distinguished from those with T1DM on the basis of concentrations of cytokines secreted by their peripheral lymphocytes stimulated with  $\beta$ -cell autoantigens, and differences in the cytokines secreted between the two variants of autoimmune diabetes. With no definite guidelines for therapy of patients with LADA to prolong insulin dependence, we speculate that these patients might have better prospects for immune therapies involving modulation of proinflammatory cytokines, like IL-17, particularly during the initial stages of the disease.

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**Conflicts of Interest:** None.

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