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TH1/TH2 cytokine balance in patients with both type 1 diabetes mellitus and asthma

Rachmiel Marianna [a], Bloch Olga [b], Bistritzer Tzvi [a], Weintrob Naomi [c], Ofan Regina [c], Koren-Morag Nira [d], Micha J. Rapoport [b,*]

[a] *Department of Pediatrics, Assaf Harofeh Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Israel*
[b] *Diabetes unit, Assaf Harofeh Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Israel*
[c] *The Institute for Endocrinology and Diabetes, Schneider Children's Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Petah Tikva, Israel*
[d] *Department of Epidemiology and Statistics, Sackler Faculty of Medicine, Tel-Aviv University, Petah Tikva, Israel*

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Abstract

Background and objective: T1DM and asthma are mediated by opposite arms of the cellular immune system namely T helper (Th)1 and Th2 $CD4^+$ cells, respectively. Our aim was to characterize the Th1/Th2 cytokine balance in patients with both T1DM and asthma.

Methods: Forty-four patients, mean age 19 years were matched by gender and age, to 4 paired groups: T1DM and asthma, asthma only, T1DM only and healthy controls. Peripheral blood mononuclear cells (PBMC) were stimulated in vitro with disease-specific recombinant antigens; glutamic acid decarboxylase and house dust mite (Der p1 antigen) for T1DM and asthma, respectively, and non-specific mitogens; phytohemaglutinin (PHA), tetanus toxin and anti-CD3 mAb. ELISPOT and ELISA technique were used to determine INF-γ, IL-2, IL-4, IL-13 and IL-10 expression.

Results: Patients with T1DM and asthma demonstrated a similar cytokine pattern but lower Th1/Th2 ratio compared to patients with T1DM only. The Th2 cytokines response to Der p1 was enhanced in patients with both diseases compared to controls. The IL-10 overall secretion was higher in patients with both diseases compared to one disease only.

Conclusion: The Th1 and Th2 secretory pattern of patients with T1DM and asthma combines features of both diseases suggesting a unique Th1/Th2 balance.
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Keywords: Type 1 diabetes mellitus; T helper cells; Cytokines; Asthma; Interleukin-2; Interleukin-4; Interleukin-10; Interferon-γ

1. Introduction

Type 1 diabetes mellitus (T1DM)[1] and asthma are chronic inflammatory diseases mediated by opposite arms of the cellular immune system namely Th1 and Th2 $CD4^+$ T helper (Th) cells, respectively [1–3]. This view of T1DM and asthma as Th1 and Th2 restricted diseases is debatable inasmuch as recent evidence suggests that Th2 cytokines are also involved in the pathogenesis of T1DM and that an increased secretion of a prototype Th1 cytokine, INF-γ is associated with severe asthma exacerbations [4,5]. Epidemiological studies that examined the co-existence of asthma and T1DM show conflicting results [6–11]. A meta-analysis reported recently demonstrated an inverse relationship between these diseases with a decreased incidence of asthma in T1DM patients [12].

* Corresponding author. Present address. Department of Internal Medicine "C", Assaf Harofeh Medical Center, Zerifin 70300, Israel. Fax: +972 8 9779285.
E-mail address: mrapoport@asaf.health.gov.il (M.J. Rapoport).

[1] *Abbreviations used:* T1DM, type 1 diabetes mellitus; Th, T helper; PBMC, peripheral blood mononuclear cells; GAD, glutamic acid decarboxylase; ELISPOT, enzyme-linked immunosorbent spot; Der p1, *Dermatophagoides pteronyssinus*.

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Limited clinical data is available regarding the phenotypic features of patients with both T1DM and asthma [13,14]. Douek et al. [13], described more wheezing attacks per year in patients with diabetes and asthma as compared to their siblings and to asthmatic patients. Wright and Wales [14] reported a decreased incidence of hypoglycemia during 3 months period in children with T1DM and asthma as compared to T1DM patients. We demonstrated recently that patients with both T1DM and asthma had reported a higher rate of hypoglycemic events compared to patients with T1DM only [15]. These data suggest that asthma and T1DM may reciprocally affect their clinical manifestations. However, whether the co-existence of these two diseases also results in a different immune balance has not been determined. The aim of our study was therefore to examine for the first time the Th1/Th2 cytokines balance in patients with both T1DM and asthma in comparison to matched patients with asthma or T1DM only and healthy controls.

2. Materials and methods

2.1. Subjects

We recruited 11 subjects who were coded to have both T1DM and asthma from two diabetic in hospital clinics; Assaf Harofeh Medical Center and Schneider Children's Medical Center. A total of 28 subjects aged 10–30 years fulfilled the inclusion criteria of T1DM and atopic asthma, however, only 11 had none of the exclusion criteria and agreed to participate. We randomly matched 11 subjects according to age and gender from the Diabetes and from the Respiratory clinics in those centers. Healthy controls were recruited from the students population attending Assaf Harofeh Medical Center and investigators friends and family members. Each group included 11 patients, 8 males and 3 females: patients with T1DM and asthma, mean age 19.2 ± 5.8 years, range 13.5–25.5 years, patients with T1DM only, mean age 19.1 ± 4.4 years, range 12.75–27.8 years, patients with asthma only, mean age 19 ± 4.3 years, range 12.5–28 years and 11 normal healthy controls, mean age 20.4 ± 4.6 years, range 11.7–25.8 years. Clinical and demographic parameters of these patients are listed in Table 1. T1DM was diagnosed according to the National Diabetes Group guidelines [16]. Only patients treated with insulin for more than a year were included. Asthma was diagnosed according to the National asthma education and prevention program guidelines [17]. All asthmatic patients had an obstructive pattern on spirometry and a positive skin test for house dust mite antigen. Patients receiving oral steroids during the study period or in the preceding 3 months or actively treated with antigenic vaccinations were excluded. Patients were also excluded if symptoms of infection or systemic somatic illness other than T1DM or asthma were present. Healthy controls had no personal or family history of atopic disorders or T1DM. All participants signed an informed consent and the hospitals ethics committees approved the study.

2.2. Clinical data

Family history, incidence of hypoglycemia defined as any documented capillary plasma glucose level below 60 mg% [18] in their chart or log book during the last 3 months, with or without clinical symptoms, insulin treatment dosage during the last 3–12 months, mean HbA1c levels during the last year, chronic renal, retinal and neurological diabetes associated complications, incidence of asthma exacerbations and usage of inhaled steroid preventive treatment for asthma within the last year were determined for all patients by review of medical records and a personal interview.

2.3. Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMC) of each patient were isolated from heparinized, freshly drawn blood samples by Ficoll-Paque™ PLUS density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). All blood samples were determined to have normal WBC profile. PBMC were washed, re-suspended, prepared at concentration 2×10^6/ml in complete cell culture medium (RPMI 1640, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine) containing 5% or 10% inactivated fetal calf serum (FCS) (Biological Industries, Beit Haemek, Israel)) and used immediately for the various assays.

Table 1
Clinical and demographic parameters of the study population

Group	Number of patients (M/F)	Age (years) (mean ± SD)	DM duration (years) (mean ± SD)	Glycated Hb (%)[a] (mean ± SD)	Insulin dosage U/kg/d (mean ± SD)	Asthma duration (years) (mean ± SD)
T1DM and asthma	11 (8/3)	19.2 ± 5.85	7.57 ± 5.46	7.71 ± 0.73	0.69 ± 0.3	15.9 ± 6.9
T1DM	11 (8/3)	19.1 ± 4.36	7.25 ± 4.68	8.54 ± 4.01	0.72 ± 0.3	—
Asthma	11 (8/3)	19 ± 4.34	—	—	—	15.1 ± 6.48
Control	11 (8/3)	20.4 ± 4.56	—	—	—	—

T1DM, type 1 diabetes mellitus; SD, standard deviation.
[a] Normal range = 4–6%.

2.4. Mitogens, nominal and specific antigens

PBMC from each patient were stimulated with the polyclonal T-cell activators: 10 μg/ml PHA (Sigma, St. Lous, USA) and 10 μg/ml anti-CD3 purified mouse monoclonal IgG (R&D systems, Minneapolis, Minnesota, USA), nominal antigen 1 Lf/ml tetanus toxoid (Pasteur Mérieux Connaught, USA) and specific antigens: 10 μg/ml recombinant T-cell GAD65 antigen (Diamid Diagnostics AB, Stockholm, Sweden) and 10 μg/ml recombinant *Dermatophagoides pteronyssinus*, Der p1 allergen (INDOOR Biotechnologies Inc., Charlottesville, USA).

2.5. Enzyme-linked immunosorbent spot (ELISPOT) assay

Human IL-2, IFN-γ, IL-4 and IL-13 Eli-spot agarose-enzymatic format Kits (Diaclone Research, France) were used for estimation of cytokine response using the corresponding manufacture protocol. Briefly, 2×10^5 cells were seeded in 100 μl medium containing 10% inactivated FCS were seeded into 96-well Maxisorp Nunc-immunoplates (Nunc, Denmark). Before cell seeding the plates were coated with capture IL-2, IFN-γ, IL-4 and IL-13 antibodies (1:100 dilution), washed and blocked with 2% BSA. Cells were then stimulated directly with the various mitogens and antigens and incubated at 37 °C in CO_2 incubator for 40 h. The appropriate secondary IL-2, IFN-γ, IL-4 or IL-13 biotinilated detection antibodies (1:100 dilution) were distributed in each well after cell lysis and incubation was proceeded for 1 h at 37 °C. Wells were washed three times, streptavidin–alkaline phosphatase conjugate (1:1000 dilution) was dispensed into wells which were incubated for 1 h at 37 °C. Plates were then washed three times, gel substrate solution (BCIP/agarose/stabilizers) was dispensed into wells, and plates were incubated at 37 °C for 2–4 h until blue spots developed. Spots were counted twice using Olympus CK2 inverted microscope and the mean of duplicate determinations for each antigen per 2×10^5 cells was calculated.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Volume of 2×10^5 cells per well were in complete medium with 5% FCS, then were seeded into round-bottomed 96-well tissue culture plates (Greiner, USA), stimulated with 10 μg/ml PHA and cultured in 5% CO_2 in a humidified 37 °C incubator. Supernatants were collected at 0, 18, 24 and 48 h after stimulation and the level of IL-10 was determined using IL-10 Eli-pair kit (Diaclone Research, France) using the manufacturer protocol. Briefly, 96-well Maxisorp Nunc-immunoplates (Nunc, Denmark) were coated with capture Ab B-N10 (1:400 dilution), washed and blocked with 5% BSA. Standards and supernatants were distributed in duplicates into precoated wells and co-incubated with detection biotinylated antibody B-T10 (1:50 dilution) for 2 h at room temperature. Wells were washed three times and incubated for 20 min at room temperature with horseradish peroxidase conjugated streptavidin (1:100 dilution). Plates were then incubated with TMB for 10–15 min in the dark and reaction was stopped by adding 1 M H_2SO_4. The optical density of each well was determined within 30 min using microplate reader set at 450 nm. CV of all cytokine assays did not exceed 10%.

2.7. Statistical analysis

Analysis of data was performed using SPSS (SPSS, version 11.0, SPSS Inc., Chicago, IL). Analysis was first performed for each group separately, using Spearman correlation. Comparisons between groups were performed by Wilcoxon's signed rank test. $p = 0.05$ was considered the limit of significance. We obtained power of 80% for detecting correlation and differences between groups.

3. Results

3.1. Th1 cytokines

INF-γ: basal non-stimulated number of INF-γ spots was significantly higher in all groups of patients and controls in comparison to all other Th1 and Th2 cytokines examined (Fig. 1). INF-γ spots generation was significantly higher in T1DM only patients than in patients with asthma only and controls in basal non-stimulated condition and following stimulations with non-antigen-specific anti-CD3 or with antigen-specific GAD and Der p1. Patients with both diseas-

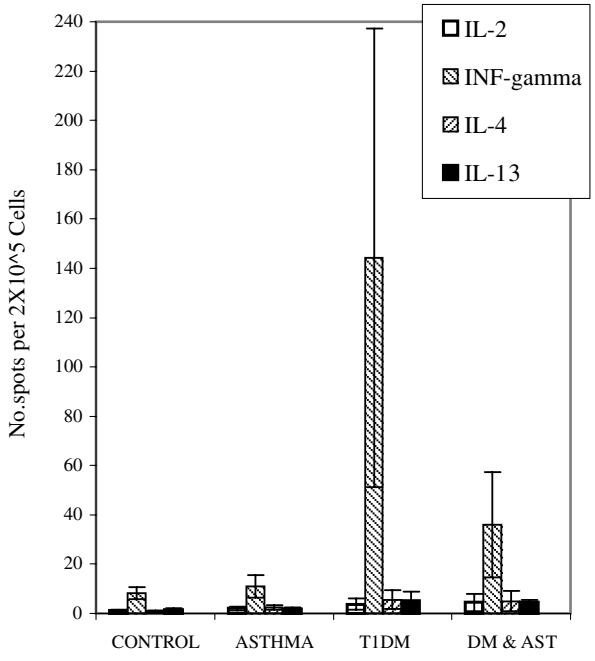

Fig. 1. Th1 (IL-2, INF-γ) and Th2 (IL-13, IL-4) cytokines expression from non-stimulated PBMC of patients with T1DM and asthma, T1DM, asthma and controls ($n = 11$ in each group), as determined by ELISPOT, presented as mean number of spots per 2×10^5 PBMC ± SEM. Basal non-stimulated number of INF-γ spots was higher than other cytokines in all groups ($p < 0.05$).

es demonstrated an intermediate INF-γ response, which was not significantly different from the other groups (Fig. 2a).

IL-2: a significant increase in IL-2 response was seen after stimulation with tetanus toxin (TT) as compared to the non-stimulated state in all participants except in patients with T1DM only who demonstrated a borderline response. Comparison among the 4 groups of patients demonstrated a significantly higher IL-2 spots generation in patients with T1DM and asthma after tetanus toxin stimulation as compared to controls ($p < 0.03$) (Fig. 2b).

3.2. Th2 cytokines

IL-4 and IL-13: spots generation of IL-4 and IL-13 was comparable in all patients' groups and controls in the non-stimulated state and following non-specific stimulation. Antigen-specific stimulation with Der p1 antigen in patients with both T1DM and asthma generated more IL-4 spots compared to controls ($p < 0.01$). A higher IL-13 spots generation of a borderline significance was also noted ($p < 0.09$) (Figs. 2c–d).

3.3. Th1/Th2 cytokines ratio

To further clarify the Th1/Th2 balance of our patients we calculated the ratio between the sum of IL-2 and INF-γ and the sum of IL-4 and IL-13 spots generation as well as the ratio between INF-γ and IL-4 spots generations. Non-specific stimulation with either PHA or anti-CD3 resulted in a significantly higher Th1/Th2 ratio in patients

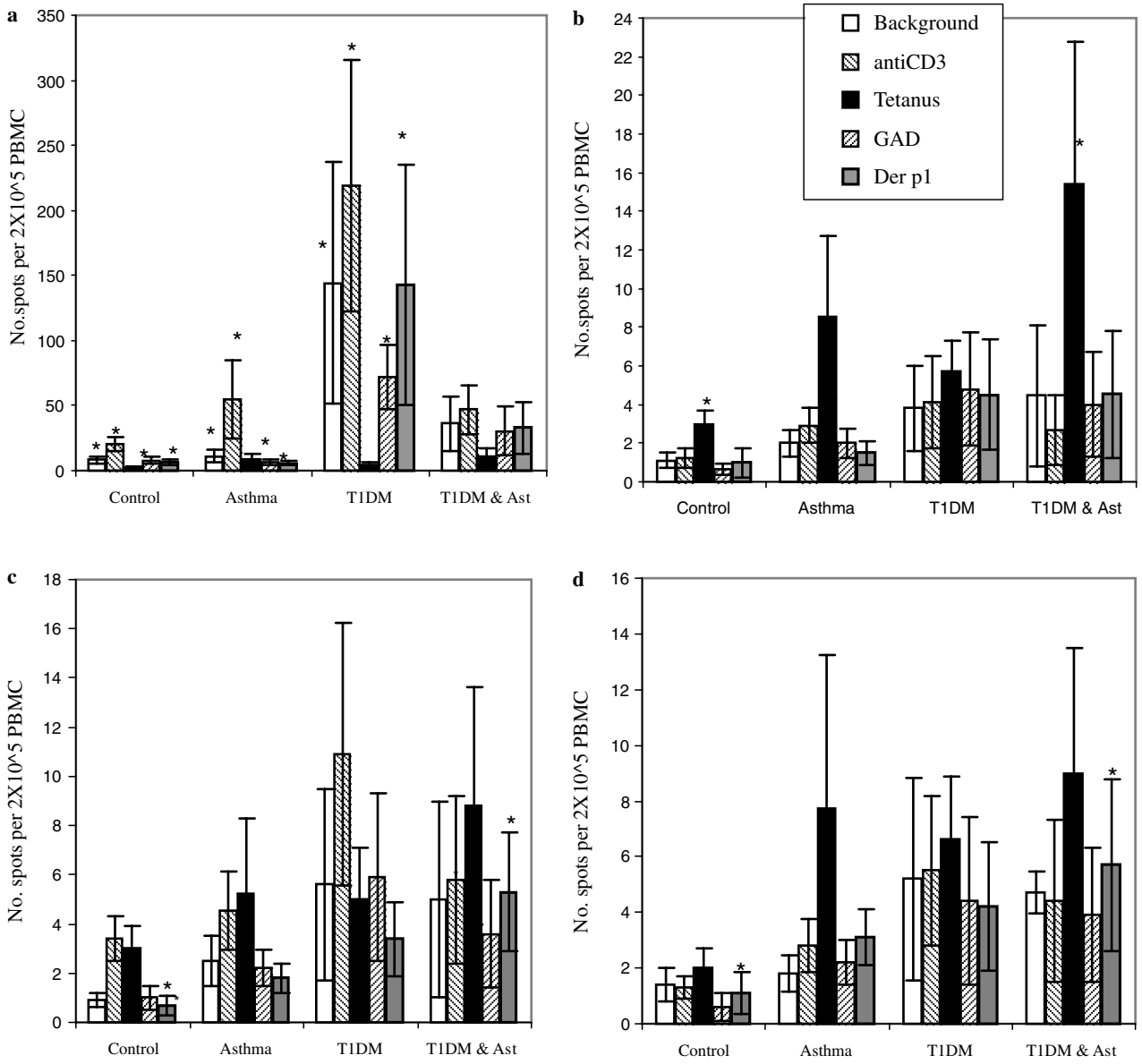

Fig. 2. Th1 and Th2 cytokines expression from non-stimulated and stimulated PBMC of patients with T1DM and asthma, T1DM, asthma and controls ($n = 11$ in each group). Determined by ELISPOT and presented as mean number of spots per 2×10^5 cells ± SEM. (a) INF-γ, $^*p < 0.05$. (b) IL-2, $^*p = 0.03$. (c) IL-4 $^*p$ value = 0.019. (d) IL-13, $p = 0.09$.

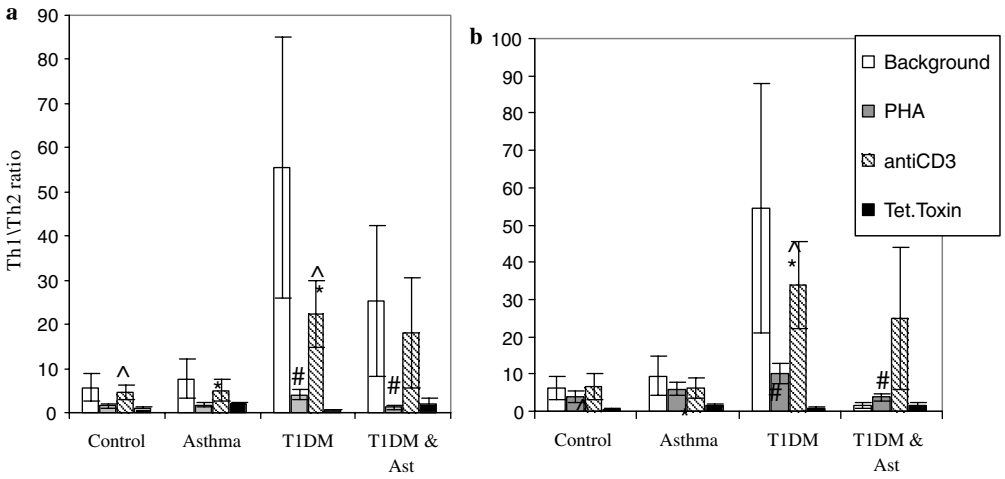

Fig. 3. Th1/Th2 cytokines ratio from non-stimulated and stimulated PBMC of patients with T1DM and asthma, T1DM, asthma and controls ($n = 11$ in each group). Determined by ELISPOT and calculated; (a) as the sum of the mean number of spots per 2×10^5 PBMC of IL-2 and INF-γ divided by the sum of the mean IL-4 and IL-13 spots. (b) As ratio between INF-γ and IL-4. $^*p = 0.009$, $^\#p = 0.02$, $^\wedge p = 0.02$.

with T1DM only as compared to all other patients' groups and controls (Fig. 3). No significant difference among the groups was observed after antigen-specific stimulation (data not shown).

3.4. IL-10

To further investigate the cytokine secretory pattern, we evaluated the non-stimulated and stimulated secretion in vitro of the regulatory cytokine IL-10. The 48 h secretory pattern of IL-10 did not differ in all patients' groups. However, the stimulated overall secretion of IL-10 in patients with both diseases was similar to that of healthy controls and significantly higher than in patients with asthma only ($p < 0.01$). It was also higher than in patients with T1DM only but this difference did not reach statistical significance (Fig. 4).

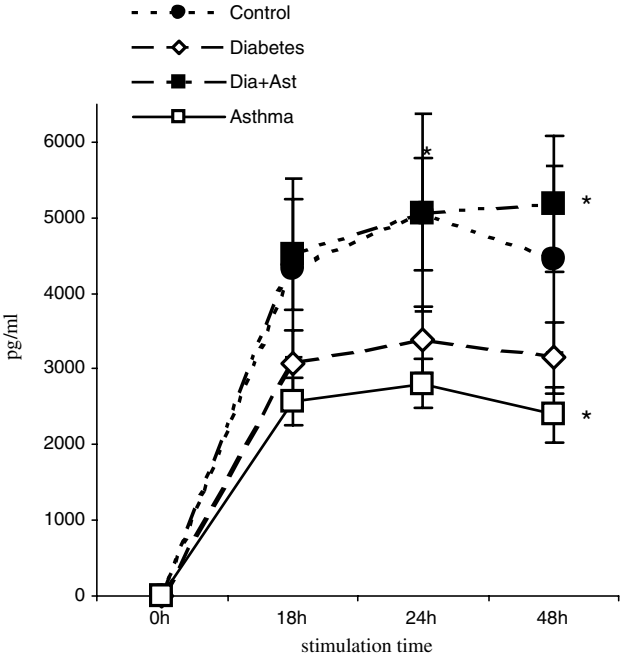

Fig. 4. Secretory profile of IL-10 of patients with T1DM and asthma, T1DM, asthma and controls. Determined by ELISA and expressed as mean ± SEM. PBMC were isolated and stimulated with PHA 10 μg/ml. Supernatants were collected at 0, 18, 24 and 48 h. Overall cytokine secretion was calculated as area under the curve (AUC) using the trapezoidal rule. $^*p < 0.01$.

3.5. Disease phenotype is not correlated to cytokine profile

No correlation was demonstrated between the cytokines secretion patterns of all patients' groups and their clinical parameters, including disease duration, frequency of hypoglycemic events or of late diabetes complications, mean HbA1c levels and frequency of wheezing events (data not shown).

4. Discussion

This study examined for the first time the balance between the Th1 and Th2 arms of the cellular immune systems occurring in patients afflicted simultaneously with both Th1 and Th2 mediated diseases. Our choice of T1DM and asthma as representative Th1 and Th2 diseases, respectively, was based on their relatively high prevalence, increasing incidence and the recent epidemiological debate regarding their co-existence [6–12]. In addition, each of these two diseases is clinically well defined, enabling non-biased patient selection and matching. To facilitate the analysis only commonly accepted T1DM associated Th1 and asthma associated Th2 cytokines were determined together with a representative regulatory cytokine namely IL-10 [19]. Our data demonstrate that patients with both diseases express a unique Th1 and Th2 cytokine pattern, which is different from patients with one disease only. The most prominent features of these patients were

observed when compared to normal controls rather than to patients with one disease only. Th1 cytokines exhibited a mixed pattern: a higher IL-2 expression following non-antigen-specific stimulation by tetanus toxin and only an intermediate INF-γ response to most specific and non-specific stimulations. The Th2 cytokine response was more uniform as both IL-4 and IL-13 responses were higher than controls when PBMC from patients with both diseases were stimulated by the specific asthma associated antigen, Der p1. The absence of these differences when comparison was made with patients with one disease only, may suggest that patients with T1DM and asthma display an intermediate cytokine phenotype combining features of both diseases. Nevertheless, the overall Th1/Th2 ratio in patients with both diseases was lower compared to patients with T1DM only and total IL-10 secretion in patients with T1DM and asthma was significantly higher than in patients with either asthma or T1DM alone. These findings underscore the unique cytokine regulatory and secretory pattern of patients with both diseases whether compared to controls or to patients with one disease only.

The ability of our patients with T1DM and asthma to mount a parallel Th1 and a Th2 response is not surprising as recent data suggest that both Th1 and Th2 cytokines may be involved in the pathogenesis of T1DM and asthma [4,20–22]. The particular cytokine pattern of patients with two diseases may be due to a newly acquired shift between Th1 and Th2 $CD4^+$ cells induced by antigenic exposure modifying the T cells microenvironment. Such a shift has been reported in in vitro culture [20] and in an animal model of experimental autoimmune encephalitis following exposure to a non-self antigen [21] as well as in NOD mice treated with the immunomodulator linomide [22]. Most importantly, it has been reported recently that autoimmune diabetes prone NOD mice can manifest an exacerbated Th2 mediated airway inflammation and bronchial hyperresponsiveness [23]. These data together with our current findings indicate that the immune system maintains its ability to mount a dual Th1/Th2 response when exposed to the appropriate antigens. They also suggest the Th1 or Th2 dominance of autoimmune diseases is adaptive and environment dependent rather than a permanently restricted cytokine phenotype. In that regard it is of interest that asthma preceded diabetes in all our patients. Whether the final cytokine balance would have been different if diabetes had been the first to occur remains an open question. Our study has several limitations including the small cohort of patients, limited number of evaluated Th1 and Th2 cytokines and the usage of two different methods, ELISA and ELISPOT, to determine the cytokine response. In addition, our in vitro data does not and in fact cannot necessarily reflect the in vivo situation, which is constantly, and dynamically changing. Thus, our findings should be interpreted with caution and require further studies examining larger number of patients with asthma and T1DM and possibly determination of diverse cytokines. In addition, examination of patients with different dual or multiple autoimmune diseases is necessary to establish the cytokine pattern resulting from the interaction of Th1 and Th2 dominant diseases.

In conclusion, this pilot study examines for the first time the Th1/Th2 cytokine secretion pattern of patients with both T1DM and asthma. Our data suggest these patients have a unique cytokine secretory pattern, which combines features of both T1DM and asthma.

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