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Abnormal Distribution of Gamma-Delta T Lymphocytes and Their Subsets in Type 1 Diabetes*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. It was assumed that T γδ lymphocytes (T γδ) are involved in the autoimmune destruction of beta cells, presumably as regulatory cells.
Objectives. The aim of this paper was to investigate T γδ cells mean percentage (%) in peripheral blood in patients with type 1 diabetes (T1DM) at the time of diagnosis and after twelve months of observation.
Material and Methods. A total of 41 patients (21 boys, 51.2%) with new-onset T1DM were included. Exclusion criteria were: Other autoimmune disease, neoplasm, and inflammation. The control group comprised of 14 healthy (7 boys, 50.0%), normostenic children, with normal glucose metabolism and negative history of autoimmune disease and/or diabetes in family.
Results. The mean T γδ % in patients with new-onset T1DM (8.03 ± 3.80) and after 12-months of follow-up (6.13 ± 2.15) was lower than in controls (11.23 ± 6.79), $p = 0.042$ and $p = 0.016$, respectively. A depletion of those cells after one year was observed ($p = 0.067$). Gender did not affect the level of T γδ and subsets. No association between T γδ and age neither in T1DM nor controls was observed.
Conclusions. Our results support the hypotheses that T γδ play a role in T1DM pathogenesis. If so, the decrease of those lymphocytes makes the probands more vulnerable to autoaggression. We report here for the first time the further depletion of T γδ after one year of insulin treatment, which may be due to the exacerbation of beta cell destruction in the course of insulitis (**Adv Clin Exp Med 2016, 25, 4, 665–671**).

Key words: immune tolerance, type 1 diabetes, T γδ lymphocytes.

The classic model of type 1 diabetes (T1DM) pathogenesis predicates that in a genetically predisposed person, a chronic autoimmune process occurs following the precipitating event, which leads to the progressive destruction of beta cells and the loss of insulin release [1]. Improper function of regulatory lymphocytes, including T γδ cells, might be crucial for initiating defected immune response, leading to self-tolerance break-down and the progressive destruction of beta cells. [2]

T γδ cells (T γδ) represent a minor subpopulation of T lymphocytes in human peripheral blood, which is 4%. They are quite numerous in the skin, the lungs and the intestinal epithelium, indicating their contribution in the immune response in foreign antigen contact sites. They are known to recognize low-weight antigens (e.g. heat shock proteins) as well as self-antigens, and reveal cytotoxic and natural killer's capabilities [3]. Most of the T γδ in the peripheral blood are Vγ9/Vδ2 cells, responsible for anti-infection properties and have an unusual capacity to activate both Th1 as well as Th2 response. They not only play a role in the defense reactions against viruses, bacteria and parasites, but also in the cytolysis of neoplastic cells. According to Caccamo et al., a dimin-

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ishing T γδ cell count with age increases the risk of autoimmune disease in the fourth and further decades of life [4].

T γδ cells are highly related to immune tolerance processes that might be impaired in autoimmune diseases [5–10]. It is probable that T γδ contribute also in islet inflammation [7, 10].

The aim of this paper was to investigate T γδ cells mean percentage (%) in peripheral blood in patients with type 1 diabetes at the time of diagnosis and after twelve months of observation.

Material and Methods

A total of 41 patients (21 boys, 51.2%) with new-onset T1DM were included into the study. Exclusion criteria were: Other autoimmune disease, neoplasm, and inflammation. Insulin therapy according to experts' recommendations was administrated and education regarding diabetes was conducted. The control group comprised of 14 healthy (7 boys, 50%) normostenic children, with normal glucose metabolism and with a negative history of autoimmune disease as well as no family history of diabetes. The clinical characteristics of both groups are presented in Table 1.

The material used for tests was 10 mL of peripheral blood drawn from the humeral vein to a test-tube containing heparin as an anti-coagulant. The quantitative and qualitative analysis of peripheral blood lymphocytes was performed by a double-color flow, using a fluorescence-activated cell sorter and monoclonal antibodies coupled with fluorescein isothiocyanate (FITC) and phycoerythrin (RPE): Ab-antiCD45-FITC, Ab-antiCD14-RPE, Ab-antiTCR gamma-delta FITC, Ab-antiCD4-RPE, Ab-antiCD8-RPE, Ab-antiCD25--RPE, Ab-antiCD56 RPE, Ab-antiCD69-RPE and Ab-antiIgG1-FITC and Ab IgG1- RPE as negative controls. The obtained counts of T γδ lymphocytes, given as percentages (%), were accepted as a measure of the population and subpopulation size of these lymphocytes in the peripheral blood. The percentage of T γδ was a sum of TCR $\gamma\delta^+$ CDX^+ and TCR $\gamma\delta^+$ CDX^- cell percentages, and was calculated from the following formula:

$$\% \text{ TCR } \gamma\delta^+ CDX^+ + \% \text{ TCR } \gamma\delta^+ CDX^- = \\ = \% \text{ TCR } \gamma\delta^+,$$

where:

– % TCR $\gamma\delta^+$: percentage of T γδ lymphocytes (whole T γδ population),

– % TCR $\gamma\delta^+CDX^+$: percentage of T γδ lymphocytes expressing the CDX antigen (T γδ CDX^+ cells) (subpopulations of T γδ $CD4^+$, T γδ $CD8^+$, and activated T γδ $CD25^+$ and T γδ $CD69^+$ lymphocytes),

– % TCR $\gamma\delta^+CDX^-$: percentage of T γδ lymphocytes not expressing the CDX antigen (T γδ CDX^- cells),

– CDX: antigen CD4, CD8, CD25 or CD69, respectively.

The size of T γδ $CD4^-CD8^-$ (T γδ "double negative") subpopulation was calculated indirectly, using the following formula:

$$\% \text{ TCR}\gamma\delta^+ CD4^-CD8^- = \% \text{ TCR}\gamma\delta^+ - \\ - (\% \text{ TCR}\gamma\delta^+CD4^+ + \% \text{ TCR}\gamma\delta^+CD8^+),$$

where:

– % TCR $\gamma\delta^+$: percentage of T γδ lymphocytes (whole T γδ population),

Table 1. Clinical characteristic of studied groups

Feature	T1DM group	Control group	p
	N = 41	N = 14	
Females/males	20/21	7/7	0.937
Age (years):	3.32 – 17.98, × = 11.41, SD = 3.81	4.92 – 17.95, × = 12.61, SD = 3.8	0.213
BMI Z-score	(–)3 – 0.81, × = (–)0.36, SD = 0.90	0.12 – 0.51, × = 0.26, SD = 0.18	0.185
Symptoms duration before diagnosis (days)	0–120, × = 29.38, SD = 33.7	–	–
DKA at presentation present (%)/no DKA	13 (31.7%)/28	–	–
%HbA1c (mmol/mol)	5.9–14.5, × = 11.63, SD = 2.23 (40.99–145.92, × = 92.80, SD = 0.87)	–	–
c-peptide at presentation (ng/mL)	< 0.05–2.13, × = 0.47, SD = 0.45	–	–
Insulin requirement (IU/kg/day)	0–2.9, × = 0.79, SD = 0.51	–	–

– % TCRγδ$^+$CD4$^+$: percentage of T γδ lymphocytes expressing the CD4 antigen (T γδ CD4$^+$ cells),

– % TCRγδ$^+$CD8$^+$: percentage of T γδ lymphocytes expressing the CD8 antigen (T γδ CD8$^+$ cells),

– % TCRγδ$^+$CD4$^-$CD8$^-$: percentage of T γδ lymphocytes not expressing both the CD4 and CD8 antigens (T γδ CD4$^-$CD8$^-$ cells).

In the study group, an examination of lymphocytes was performed twice: At the diagnosis and after 12 months of treatment. Ten patients (24.4%) were excluded from this part of the study due to loss of observation. In the control group one-time examination of lymphocytes was performed.

The Ethic Committee at the Wroclaw Medical University obtained positive opinion and informed consents of patients and legal guardians were obtained.

Statistical Analysis

Statistical analysis was performed using the STATISTICA 9.1 (StatSoft Inc., Tulsa, USA). The following statistical measures were used: Arithmetical mean (x) and standard deviation (SD). Moreover, minimal (min) and maximal (max) values of studied parameters were given.

The variable distribution was evaluated by the Shapiro-Wilk test. For variables with normal distribution, Student's t-test was used when comparing two independent samples and paired samples, and t-test was used for dependent ones. For variables with distribution other than normal, Mann-Whitney U test for independent variables and the Wilcoxon signed-rank test for related samples were used. The results with a significance level of $p < 0.05$ were considered statistically significant, and if $0.05 \leq p < 0.1$ – the difference was considered to be on the border of statistical significance.

In order to calculate the relationship between studied parameters, Pearson correlation coefficient (for variables with normal distribution) or Spearman's rank correlation coefficient (for non-parametric tests) were used.

Results

The T γδ lymphocytes % in the peripheral blood of patients with new-onset T1DM as well as after 12-months of treatment was lower ($p = 0.042$, $p = 0.016$, respectively) than in the control group. Moreover, we observed further depletion of the percentage of Tγδ cells in the patients' group ($p = 0.067$) (Table 2).

The analysis of Tγδ subsets in the peripheral blood revealed that the depletion of these cells in T1DM patients was due to a smaller ($p = 0.01$) size of T γδ CD4$^-$CD8$^+$ subpopulation. This was true both at the onset of the disease and after one year of treatment. The level of T γδ CD4$^+$CD8$^-$ was similar to the percentage in the control group. Moreover, the level of "double negative" Tγδ CD4$^-$ CD8$^-$ cells was significantly smaller ($p = 0.02$) only in the treated patients. Detailed data is presented in Table 3.

At the onset of diabetes, the sizes of activated subpopulations T γδ CD25$^+$, T γδ CD56$^+$ and T γδ CD69$^+$ in the peripheral blood of patients were similar to the controls; however, a trend was marked that the level of T γδ CD25$^+$ was higher in T1DM group (Fig. 1). In the same group after 12 months of treatment, the sizes of T γδ CD25$^+$ and T γδ CD56$^+$ subsets depleted (Table 4).

The level of T γδ and subsets was similar in boys and girls, both in patients and controls, $p > 0.05$. They were also similar for boys with T1DM and healthy ones ($p > 0.05$), whereas in females the mean percentages of T γδ as well as Tγδ CD4$^-$CD8$^+$ were significantly lower in patients: 3.93–23.13, $x = 7.84$, $SD = 4.29$ vs. 4.10–27.34, $x = 13.94$, $SD = 9.54$, $p = 0.039$ and 0.04–1.64, $x = 0.56$, $SD = 1.20$, $p = 0.004$, respectively.

We did not observe the association between Tγδ and age neither in T1DM group nor controls; however, the Tγδ CD4$^+$CD8$^-$, Tγδ CD56$^+$ and Tγδ CD69$^+$ subpopulations correlated with age of the patients: $r = (-)0.36$, $p = 0.038$; $r = 0.45$, $p = 0.007$ and $r = (-)0.38$, $p = 0.024$, respectively.

Table 2. The T γδ lymphocytes percentages in peripheral blood of T1DM patients at the time of diagnosis and after 12 months of treatment and in control group

Group		% of T γδ cells in peripheral blood					
		N	range of values		x	SD	p
			min	max			
I	T1DM at diagnosis	41	0.45	23.13	8.03	3.80	$p_{I:III} = 0.042$
II	T1DM – after 12 months of treatment	31	2.54	9.71	6.13	2.15	$p_{I:II} = 0.067$
III	Controls	14	4.1	27.34	11.23	6.79	$p_{II:III} = 0.016$

Table 3. The T γδ subpopulations in peripheral blood of T1DM patients at diagnosis and after 12 months of treatment and in control group according to CD4 and CD8 antigens expression

T γδ cells subpopulation		**A** **TCR γδ** **$CD4^-CD8^-$**	**B** **TCR γδ** **$CD4^-CD8^+$**	**C** **TCR γδ** **$CD4^+CD8^-$**	**p**
I T1DM at diagnosis	min–max	0.400–21.640	0.020–1.640	0.000 – 0.180	$p_{A:B} < 0.0001$ $p_{A:C} < 0.0001$ $p_{B:C} < 0.0001$
	x	8.118	0.569	0.063	
	SD	2.031	0.263356	0.079671	
II T1DM, after 12 months of treatment	min–max	2.200–8.960	0.000–0.760	0.000–0.230	$p_{A:B} < 0.0001$ $p_{A:C} < 0.0001$ $p_{B:C} = 0.0051$
	x	5.552	0.327	0.092	
	SD	2.031	0.263	0.080	
III Controls	min–max	2.440–25.160	0.170–3.040	0.000–0.210	$p_{A:B} < 0.0001$ $p_{A:C} < 0.0001$ $p_{B:C} = 0.0005$
	x	9.967	1.068	0.071	
	SD	6.183	0.937	0.061	
p		$p_{I:II} = 0.069$ $p_{II:III} = 0.021$ $p_{I:III} = 0.265$	$p_{I:II} = 0.065$ $p_{II:III} = 0.011$ $p_{I:III} = 0.012$	$p_{I:II} = 0.134$ $p_{II:III} = 0.465$ $p_{I:III} = 0.602$	

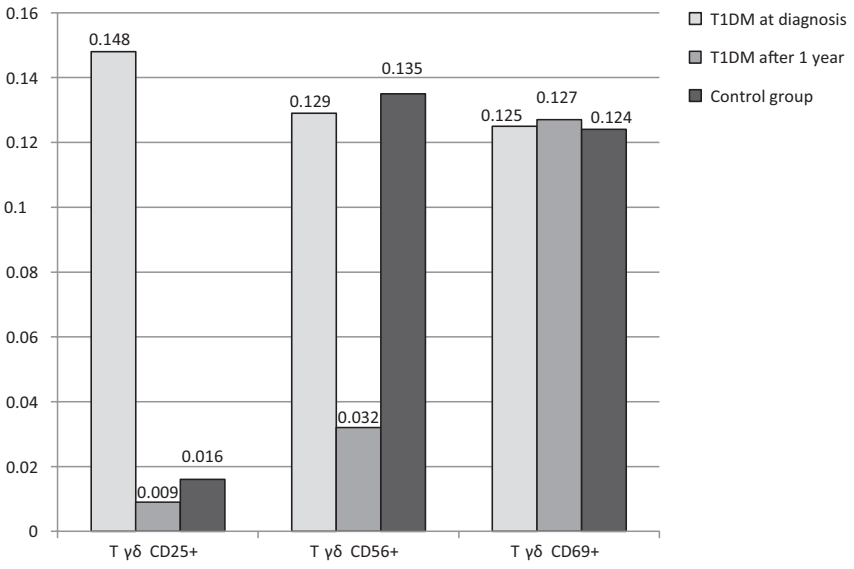

Fig. 1. Mean percentages of activated T γδ lymphocytes: T γδ $CD25^+$, T γδ $CD56^+$ and T γδ $CD69^+$ in the peripheral blood of T1DM patients and control group

Discussion

Even though studies on the role of T γδ lymphocytes in type 1 diabetes started in the mid-nineties of the past century, their true meaning in the autoimmune diseases, including type 1 diabetes pathogenesis, is still unrevealed. Due to differences in in the structure of both gamma and delta chains of TCR that determines cell function: Cytotoxic or regulatory, T γδ cells in the immune response may act pro- as well as anti-inflammatory [3]. The alterations in the function and size of the population of T γδ are reported for numerous autoimmune diseases, e.g. multiple sclerosis, lupus erythematous and celiac disease [4, 9–12].

According to Borst and Lafont, the size of T γδ population in healthy Caucasian adults did not exceed 5%, and according to Groh et al. ranged between 0.5 and 16% [13–15]. In the Hviid study, the percentage of T γδ was comparable in children and adults, not exceeding 4% in Caucasians and 10% in Africans [16]. In healthy Polish children examined by Szczepańska, the percentage of T γδ cells ranged from 0.6 to 17%, did not differ between genders and was not influenced by age [17].

In the present study, the size of T γδ populations in T1DM group ranged between 0.45% and 23.13%, was significantly ($p = 0.042$) lower than in healthy controls (4.1%–27.34%) and comparable to the level reported by Ernerudh [18]. Our observa-

Table 4. The activated Tγδ lymphocytes subpopulations: Tγδ CD25$^+$, Tγδ CD56$^+$ and Tγδ CD69$^+$ in peripheral blood of T1DM patients at diagnosis and after 12 months of treatment and in control group

T γδ cells subpopulation		**A** **TCR γδ** **CD25$^+$**	**B** **TCR γδ** **CD56$^+$**	**C** **TCR γδ** **CD69$^+$**	**p**
I T1DM at diagnosis	min–max	0.00–4.530	0.00–0.720	0.00–0.750	$p_{A:B} = 0.881$ $p_{A:C} = 0.856$ $p_{B:C} = 0.838$
	x	0.148	0.129	0.125	
	SD	0.752	0.190	0.143	
II T1DM – after 12 months of treatment	min–max	0.00–0.050	0.00–0.230	0.00–0.630	$p_{A:B} = 0.216$ $p_{A:C} = 0.015$ $p_{B:C} = 0.061$
	x	0.009	0.032	0.127	
	SD	0.016	0.064	0.162	
III Controls	min–max	0.00–0.080	0.00–0.460	0.00–0.440	$p_{A:B} = 0.009$ $p_{A:C} = 0.002$ $p_{B:C} = 0.838$
	x	0.016	0.135	0.124	
	SD	0.025	0.156	0.115	
p		$p_{I:II} = 0.511$ $p_{II:III} = 0.434$ $p_{I:III} = 0.516$	$p_{I:II} = 0.082$ $p_{II:III} = 0.036$ $p_{I:III} = 0.912$	$p_{I:II} = 0.964$ $p_{II:III} = 0.961$ $p_{I:III} = 0.991$	

tion could be explained by studies showing in NOD mice that a decreased level of T γδ cells caused diabetes development and the expansion of T γδ CD4$^-$ CD8$^+$ cells after aerosol insulin treatment was highly protective against auto aggression [6, 19, 20]. The structure of γδ TCR enables these cells to reside in the mucosa of nasopharynx and the gastrointestinal tract that results in "easier" access to various antigens (in the cited studies – to nasal or oral insulin) and increases the possibility to raise the immune tolerance for those antigens [19, 20]. A study by Goldrath et al. showed that in NOD mice proportions of T γδ lymphocytes, "double positive" T γδ CD4$^+$CD8$^+$ and "double negative" T γδ CD4$^-$CD8$^-$ were higher amongst cells infiltrating lymph nodes in comparison to peripheral blood [21].

The results of our investigations are in agreement with Krętowski et al. who compared the T γδ cells in patients with T1DM and their relatives positive for autoantibodies (the prediabetes group) [22]. The T γδ percentages in the diabetes group and in prediabetes were both lower than in controls; however, the differences were not statistically significant. In relatives ICA and/or GADA positive with impaired insulin release, the T γδ level was significantly smaller than in the controls [22].

The results of studies by Lang et al. were ambiguous. They reported higher percentages of T γδ lymphocytes in relatives of T1DM patients, positive for ICA, due to the increase of Vγ9/Vδ2 subset. Populations of T γδ cells in new-onset T1DM patients, relatives negative for autoantibodies and healthy controls were similar [5]. On the other hand, they stated that in patients with high risk of developing diabetes (ICA positive and impaired insulin release), the T γδ level was smaller in comparison to ICA positive group with normal insulin release [5]. A follow-up examination of this group has shown that the depletion of T γδ population correlated with further insulin release impairment and diabetes manifestation in "high-risk" patients, whereas in persons in whom the percentage of studied lymphocytes were stable, beta cells function remained normal. In this aspect, our results are consistent with Lang studies, who also proved that T Vγ9/Vδ2 are regulatory cells acting in diabetes type 1 development and may be monitored as a prognostic factor for diabetes manifestation [5]. Both Lang as well as Krętowski had shown that in prediabetes patients with the highest percentages of T γδ cells, the insulin response for IVGTT was normal [5, 22].

Investigations conducted in other autoimmune diseases had shown an infiltration of T γδ lymphocytes to affected organs, like synovial fluid and synovial membrane in rheumatoid arthritis, skin in systemic sclerosis and peripheral tissues in systemic lupus erythematous (SLE). Such redistribution of T γδ lymphocytes caused their decrease in the peripheral blood. Indeed, a smaller number of T γδ cells in peripheral blood of SLE patients in comparison to healthy donors was reported by Robak et al. [12]. What is more interesting, in patients with neoplastic disorders, the percentage of T γδ lymphocytes in peripheral blood is lower than in healthy controls [23, 24]. According to lymphocytes' migration hypothesis by Zocchi, those cells are attracted by neoplastic antigens and expand

to a tumor site, and act as a first-line antitumor defense thanks to cytolysis capability [23]. It seems that our results, together with others in various autoimmune diseases, could favor this hypothesis in disorders from auto-aggression. However, in the present study, we did not perform an evaluation of lymphocytes in the biopsy material.

Both in children with T1DM and in healthy controls, the largest subset of T γδ lymphocytes were "double negative" $CD4^-CD8^-$ that was 93% and 90%, respectively. Cytotoxic/suppressor $CD8^+$ cells comprised 6% of all lymphocytes in T1DM group and 9% in controls, whereas T γδ $CD4^+$ counted only 1% in both groups. The distribution of particular subpopulations of T γδ in our observation is consistent with earlier reports by Szczepanik et al. where in healthy population TCR γδ $CD4^-CD8^-$ and TCR γδ $CD4^-CD8^+$ cells were in the majority, whereas Tγδ $CD4^+CD8^-$ in minimal [25]. Moreover, our investigations showed a significant ($p = 0.012$) decrease of T γδ $CD8^+$ subset in patients with new-onset T1DM when compared with healthy controls. This is in agreement with the study of Krętowski et al. who found that percentages of T γδ $CD8^+$ were similar in diabetic probands, ICA-positive relatives and in control group, but significantly lower in patients with prediabetes with impaired insulin secretion [22]. The depletion of T γδ lymphocytes, both $CD8^+$ and $CD8^-$ were shown also in NOD mice studies [19].

Surface molecules CD25, CD56 and CD69 are markers of T lymphocytes activation. CD56 molecule is characteristic for NK cells, in previous studies cytotoxic activity of T γδ $CD56^+$ against multiple myeloma and B-cell lymphoma was demonstrated [26]. CD69 molecule takes part in activation of cytokine production, including interleukin-2 and its receptor – CD25 molecule. CD25 molecule is a marker of regulatory lymphocytes [27]. In lymphocyte activation process, CD69 antigen appears earlier than in CD25; therefore, it is called 'early T lymphocytes activator', whereas CD25 is called 'late T lymphocytes activator' [28]. In the present study, the sizes of activated T γδ cells subpopulation: T γδ $CD25^+$, T γδ $CD56^+$ and T γδ $CD69^+$ did not differ significantly between groups. It is, however, worth noting that in new onset type 1 diabetes the percentage of T γδ $CD25^+$ was higher not only than in controls, but also in T1DM group after 12-monts of treatment. This might be due to the unusual stimulation of the immune response at the time of diabetes manifestation and in order to stop the destruction of beta cells, but this hypothesis needs to be verified in further studies.

Analysis by Cairo et al. in an adult healthy population showed that T γδ cell count in males and females are similar, identical to that of Polish boys and girls [17, 29]. In the control group, T γδ lymphocytes percentage was not significantly higher in females than in males, and T γδ $CD4^-CD8^+$ cells subset was bigger in girls vs. boys, with a difference on the border of significance ($p = 0.067$). Moreover, we found a significant decrease of T γδ (with $p = 0.039$) and T γδ $CD4^-CD8^+$ (with $p = 0.004$) in T1DM females vs. healthy females. We did not report such differences in boys. This might indicate that T γδ and T $CD4^-CD8^+$ alterations are some, but not all, impaired modes one, but not only, impaired modes in the whole immunity machinery.

We did not find the correlation between the whole population of T γδ cells and age, neither in patients, nor in healthy people. In the T1DM group, however, the negative correlation between age and T γδ $CD4^+CD8^-$ as well as T γδ $CD69^+$, and a positive correlation between age and T γδ $CD56^+$ were found. Those findings are in agreement with Lang, whereas Krętowski pointed out such association in controls [5, 22].

To summarize, our results concerning T γδ cells population in type 1 diabetes correspond with the previous hypotheses that T γδ, and especially T γδ $CD8^+$ subset, play a role in type 1 diabetes pathogenesis, presumably as regulatory cells. If so, the decrease of those lymphocytes makes the probands more vulnerable to auto-aggression, including type 1 diabetes. We reported here for the first time the further depletion of T γδ, T γδ $CD8^+$ and T γδ $CD25^+$ cells after one year of insulin treatment that may be due to termination of beta cell destruction in the course of insulitis.

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