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Increased Level of Interleukin-17 in Children and Adolescents with Type 1 Diabetes Mellitus and its Association with Vitamin D Deficiency

Tip 1 Diabetes Mellitus'lu Çocuk Ergen Hastalarda Artmış İnterlökin 17 ve Bu Artışın D Vitamini ile İlişkisi

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Abstract

Introduction: The up-regulation of interleukin-17 (IL-17) has been reported to be the pathogenesis of type 1 diabetes mellitus (T1D). On the other hand, vitamin D deficiency is fairly prevalent in T1D. This study aims to investigate IL-17 and vitamin D status and its correlation in children and adolescents with T1D.

Material and Methods: A cross sectional study was carried out between January to March 2017. A total of 20 patients aged 12.25±3.74 years old with T1D and 20 healthy control group were involved. Their parents signed an informed consent. Demographic data were obtained using structured questionnaires. Physical, and laboratory examination were also performed. Blood samples were collected and serum vitamin D and IL-17 levels were measured by indirect ELISA were.

Results: The serum level of vitamin D serum and IL-17 level between both groups were significantly different ($p<0.001$). A positive correlation between vitamin D and IL-17 levels ($p<0.05$; $r=+0.566$) was found.

Conclusion: T1D is a T cell-mediated autoimmune disorder which targets and destroys insulin-producing pancreatic beta-cells. Children and adolescents with T1D show an increased level of IL-17 immunity and vitamin D deficiency. IL-17 immunity control and vitamin D supplementation could be potential targets for further development of T1D therapeutic strategies.

Key Words: Type 1 Diabetes Mellitus, Interleukin-17, Vitamin D

Öz

Giriş: Tip 1 Diabetes Mellitus (T1D)'un patogenezinde İnterlökin 17(IL-17)'nin artışının rolü olduğu bildirilmiştir. Öte yandan, T1D'lu hastalarda çoğunlukla D vitamini eksikliği saptanır. Bu çalışmada amaç, çocuk ve ergen T1D'li hastalarda IL-17 ve D vitamininin düzeylerini araştırmaktır.

Gereçler ve Yöntemler: Çalışmada, 2017 Ocak ayı ile 2017 Mart ayı arasındaki hastalar çapraz kesitsel olarak irdelendi. Yaşları 12.25±3.74 arasında olan toplam 20 T1D'li olgu ile 20 sağlıklı birey kontrol grubu olarak çalışmaya dahil edildi. Hastaların ebeveynleri çalışmaya katılmak için bilgilenmiş onay verdi. Olguların demografik verileri oluşturulmuş anket formlarının doldurulması ile elde edildi. Ayrıca fizik muayene bulguları ve laboratuvar değerleri kaydedildi. Olgulardan kan alınarak indirekt ELISA yöntemi ile kan D vitamini ve IL-17 seviyeleri ölçüldü.

Bulgular: D vitamini ve IL-17 seviyeleri 2 grup arasında karşılaştırıldığında istatistiksel olarak anlamlı derecede farklılık gözlendi ($p<0.001$). Vitamin D ve IL-17 seviyeleri arasında pozitif bağlantı saptandı ($p<0.005$; $r:+0.566$)

Sonuç: T1D, T lenfositlerinin pankreasta insülin salgılayan beta hücrelerini hedefleyerek harap ettiği T-hücresine bağlı bir otoimmün hastalıktır. Çocuk ve erişkin TID hastalarında IL-17 düzeylerinde artış, D vitamini düzeylerinde azalma gözlenmiştir. T1D'nin tedavisinde IL-17'ye bağlı immünitenin hedeflenmesi ve hastalara D vitamini verilmesi önerilebilir.

Anahtar Kelimeler: Tip 1 Diabetes Mellitus, İnterlökin-17, D Vitamini

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Introduction

Diabetes mellitus is one of the most common metabolic diseases with high morbidity and mortality in the world. The International Diabetes Federation (IDF) reported that 425 million people in the world live with diabetes in 2017; and it is estimated that the number will rise to 629 million in 2045.[1] Meanwhile, Indonesian five-yearly basic

health research conducted by the Ministry of Health reported that 6.9% or 12 million people in Indonesia have diabetes mellitus in 2013.[2] Furthermore, World Health Organization (WHO) showed that Indonesia is ranked seventh in the world for prevalence of diabetes mellitus in 2015, after China, India, US, Brazil, Russia, and Mexico.[3] The study also showed that mortality rate of diabetes mellitus in Indonesia is ranked second in the world after Sri Lanka.

Diabetes mellitus is classified by the American Diabetes Association (ADA) into four types. Type 1 diabetes mellitus (T1D) is due to β-cells destruction, usually leading to absolute insulin deficiency. T1D is the most common type of diabetes mellitus in children and adolescents.[4] The incidence of T1D has been increasing worldwide.[5] Furthermore, IDF has estimated that globally 78,000 children develop T1D every year. As of 2017, an estimated 1.1 million of children and adolescents have T1D worldwide, and 132.6 hundred of newly diagnosed cases are reported each year.[1] Another study from Indonesian Pediatrician Association reported 731 patients with T1D in 2012. A similar study in pediatric unit of Saiful Anwar General Hospital Malang, Indonesia reported 35 patients with T1D in 2013.[6]

The pathogenesis of autoimmune destruction of beta-cells are not fully understood, but multiple genetic predispositions and relation to environmental factors have been implicated.[7] Genetic predispositions mainly occur in individuals with either HLA-DR3/4 DQ8 genotype, or both; however, an environmental factor is necessary as a trigger.[8,9] Recently, T1D development and progression is associated with the inflammation of the exocrine pancreatic tissues.[10,11] Hyper-expression of class I major histocompatibility complex (MHC) gene (*H-2Kb*) in the β-cells and the presence of auto antigen-specific CD8 T cells in pancreatic islets had been reported.[10,11,12] Inflammation of Langerhans' islets in the pancreas marked by infiltration of T lymphocyte cause an increase in reactive oxygen species generation in acinar cells contributing to β-cell destruction.[9,13] CD4 T cells, B cells, natural killer cells, and macrophages can also be found in insulitic lesions which are thought to participate in β-cell destruction through the release of inflammatory mediators that trigger β-cell apoptosis.[14] The infiltration of CD8 and CD4 T cells might induce the initiation and propagation of diabetes. The presence of CD8 T cells are not only antigen-driven; tissue-resident memory T cells can also permanently reside in peripheral tissues which occur due to local inflammation.[11]

Meanwhile, autoantibodies and B lymphocytes may contribute to the pathogenesis of T1D.

Patients with T1D commonly produce autoantibodies to islet cell cytoplasm (ICA), native insulin (insulin autoantibodies, or IAAs), 65-kDa isoform of glutamic acid decarboxylase (GAD65), the insulinoma antigen 2 protein (IA-2), and variants of zinc transporter 8 (ZnT8).[15,9] Those immune response signaling play the most significant role in the initiation of pancreatic beta-cells destruction.[16]

The inflammation process in Langerhans' islets of pancreas has been shown to destruct pancreatic beta-cells leading to progressive loss of insulin production due to dendritic cells (DCs) as antigen presenting cell, and T lymphocyte infiltration.[17] T helper 1 (Th1), Th17, Th22, and Th9 subsets from T cells lymphocyte generally drive pathogenic effector responses, whereas Th2, $CD4^+CD25^+$ forkhead box p 3 (Foxp3)+ T regulatory cell (Treg), interleukin-10 (IL-10)-secreting regulatory T cell (Tr1), and transforming growth factor (TGF)-β-secreting T cell (Th3) subsets mediate regulatory responses.[18] IL-17 which is secreted by Th17-related transcription factor RORC2 and IL-22 cytokine are increased in T1D with CCR6 expression.[19] Several studies showed up-regulation of Th17 immunity in peripheral blood T cells in T1D.[19,20] Activation of IL-17 pathway accelerates Langerhans' islets of pancreatic beta-cells apoptosis, and lead to autoimmune diabetes.[21]

Those adaptive immune response is regulated by endocrine mechanism through immune-modulatory effects of vitamin D.[22] Vitamin D is a fat-soluble vitamin that is found in dietary supplement, and also produced endogenously. Solar UV-B irradiates 7-dehydrocholesterol present in the skin to generate cholecalciferol. Activation of cholecalciferol requires hydroxylation in the liver (25-hydroxylases) and kidney (1a-hydroxylase to $1.25(OH)_2D_3$). Active hormonal form of vitamin D ($1.25(OH)_2D_3$) binds and activates the vitamin D receptor (VDR), a nuclear receptor present in nearly all nucleated cells. $1.25(OH)_2D_3$ protects the β-cells from damaging immune attacks, both directly on the β-cells which have receptors for $1.25(OH)_2D_3$ (VDR) and indirectly by acting on different immune cells, including inflammatory macrophages, dendritic cells, and T-cells.[23] Active vitamin D modulated in the immune cascade

by dendritic cell (DCs) as the main target to produce less pro-inflammatory and more anti-inflammatory cytokines. Active form of Vitamin D has anti-inflammatory effect and has an ability to down-regulate the expression and production of several pro-inflammatory cytokines.[24] Moreover, vitamin D is important in the prevention of islet cell death and improvement of insulin production.[25] Thus, vitamin D has been implicated in T1D. Recent studies to prevent the destruction of pancreatic beta-cells are more challenging. Thus, we aimed to show the important role of vitamin D and IL-17 serum level, also the correlation between vitamin D and IL-17 in children and adolescents with T1D. Further, we aspire to aid in the development of a novel potential therapeutic strategy for T1D based on the control of IL-17 immunity and vitamin D serum level.

Material and Methods

Patients and Control Subjects

Our research complied with the Helsinki Declaration. All patients and parents signed an informed consent form before participation. Patients were recruited from outpatient clinic of the Pediatric Endocrinology Clinic, Department of Pediatrics, Saiful Anwar General Hospital Malang Indonesia in the period of January to March 2017. All new patients are aged from 1 to 18 with clinical and laboratory diagnosis of T1D participated according to ADA definition. Those who presented any of the following conditions were excluded: autoimmune diseases (Systemic lupus erythematous, Noonan Syndrome), infectious diseases (tuberculosis, sepsis, HIV), abnormal liver and renal function, and history of previous vitamin D therapy for at least 1 month.

Control group consists of healthy children. Those who presented any of the following conditions were excluded: abnormal liver and renal function, previous history of diabetes, previous vitamin D therapy for at least 1 month, and autoimmune diseases.

All blood samples were collected during the initial visit, and the serum level of vitamin D (25(OH)D), and Interleukin-17 were measured in both groups. The study protocol was approved by the medical ethics committee of Saiful Anwar General Hospital. The ethical clearance was approved by Regional Medical Sciences Research Ethics Committee of Faculty of Medicine, Brawijaya University. All patients were on insulin treatment, based on standard therapy for T1D.

Vitamin D ($1.25(OH)_2D_3$) Serum Level Measurement

Serum level of circulating vitamin D ($1.25(OH)_2D_3$) was measured using enzyme-linked immunosorbent assay (ELISA) by means of Alegria Human Vitamin D ELISA Kit (ORG 270), made by Orgentec. Samples (serum or plasma), plus EDTA or heparin, were collected in a polypropylene tube and stored at -20°C. Each tube was grouped for control and sample. One tube as a calibrator was prepared. 25-D biotin solution was added to all tubes. The tubes were then centrifuged 200 rpm for 10 seconds, then 200 µL of dilution calibrator was added to each well and covered with adhesive strip. After antigen had been coated to micro plate, it was incubated at 18–25°C for two hours. All wells are washed three times with wash Buffer solution. Subsequently, 200 µL of enzyme conjugate was added to the well using a multichannel pipette. The plate was then closed and wrapped in plastic and incubated at 18–25°C for 30 minutes. Tetra Methyl Benzidine (TMB) substrate of 200 µL was added to all wells, the plate was covered by a new adhesive strip, incubated 18–25°C for 30 minutes. 100 µL substrate solution was added to all wells. Absorbance of each well was measured at 650 nm using a micro-plate reader within 30 minutes after the addition of a stop solution. The result was read by observing the optical density at 450 nm using plate reader (normal circulating vitamin D level>30 ng/mL, vitamin D insufficiency=20–30 ng/mL, vitamin D deficiency<20 ng/mL).

Interleukin-17 Serum Level Measurement

Serum level of IL-17 was measured in pg/mL by means of Human IL-17 Quantikine ELISA kits (R&D Systems, US). All standard reagents and samples were prepared. Blood samples were centrifuged, then the supernatant is diluted using Assay buffer with Flexion 1:10. The results were then put into the ELISA micro-plate and incubated at 4°C overnight. Cash samples were suspended with PBS-Tween three times each for 5 minutes. As many as 50 µL of blockage buffer in the form of 1% Bovine Serum Albumin (BSA) was added to Phosphate Buffer Saline (PBS) for 45 minutes. Reagent was then washed with PBS-Tween three times each for 5 minutes. Sample was then incubated with 100 µL of primary antibody under 1% PBS-BSA with a ratio of 1:500 for 2 hours. Repayment with PBS-Tween three times each for 5 minutes. Secondary antibody incubation in saline buffer/buffer saline with

a ratio of 1:2500 for 90 minutes. Suspension with PBS-Tween two times each for 5 minutes. 50 µL of para-Nitrophenylphosphate substrate (pNPP) was added, and incubated for 30 minutes. Reactant are washed by PBS-Tween two times, each for 5 minutes. TMB substrate was added and incubated for 30 minutes. The reaction was ended by adding Sodium Hydroxide (NaOH) 1 N for 15 minutes. The results were read in the ELISA reader with a wavelength of 450 nm, then the absorbance obtained using the standard curve of IL-17 in units of pg/mL.

Statistical Analysis

Statistical analysis was conducted by SPSS software (17.0). For the continuous variables, the Kolmogorov-Smirnov normality test was used. Quantitative data was expressed as mean ±2 standard deviation (SD). Statistical significance of quantitative variables between different categories was analyzed using independent sample t-test. Pearson's correlation coefficient (r) was used to indicate significant linear relationship among quantitative variables. Data was analyzed at 95% confidence interval (α=0.05).

Results

We studied 20 children with T1D (6 boys and 14 girls), with mean age of 12.3±3.7 years old and mean duration of diabetes 4.5±2.4 years. A control group of 20 children (9 boys and 11 girls) with mean age 11.0±2.5 years old were also involved for comparison of vitamin D serum level and IL-17 immunity in peripheral blood between diabetic and healthy children. The characteristics of the patients and controls are given in Table 1. All subjects in the T1D group had positive antibody of GAD65 and mean insulin need were 1.285 IU/kg/day SD with blood glucose monitoring as many as 1–2 times/day.

Table 1. Characteristic of subjects

		Patients with T1D	Healty control
Age (year)		12.3±3.7	11.0±2.5
Sex	Male	6	9
	Female	14	11
Duration of T1D (years)		4.5±2.4	-
Insulin dose (IU/kg/day)		1.29±0.26	-
Positive autoantibody GAD65		All patients	-
Vitamin D serum level (ng/ml)		20.14±5.87*	33.86±3.64
IL-17 (pg/ml)		146.18±45.50	11.21±75.38

*p<0.001 compared to healthy controls

Vitamin D ($1.25(OH)_2D_3$) and Interleukin-17 Levels

Vitamin D (33.86±3.64 vs 20.14±5.87) (Figure 1) and IL-17 (11.21±5.38 vs 146.18±45.49) (Figure 2) between control and T1D group were significantly different (p<0.001). The level of serum vitamin D in the T1D group was lower than the control group whereas the level of IL-17 in the T1D group was higher than the control group.

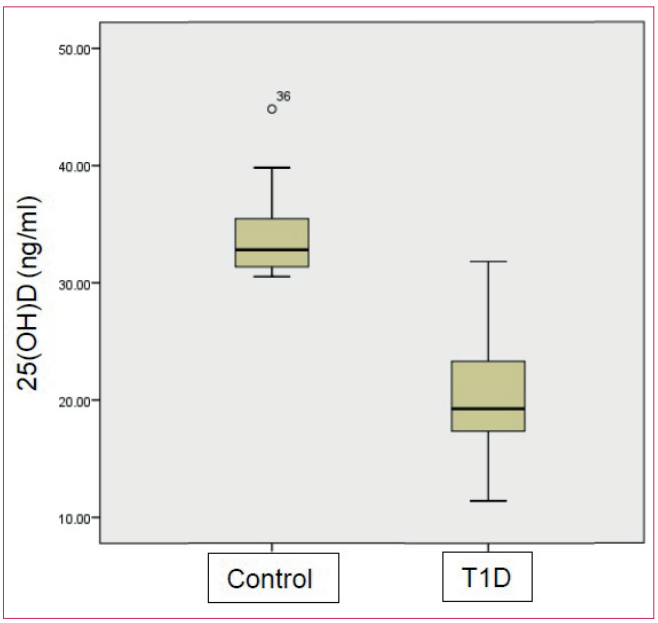

Figure 1. Mean vitamin D 25(OH)D levels in control and patients with T1D group p<0.001

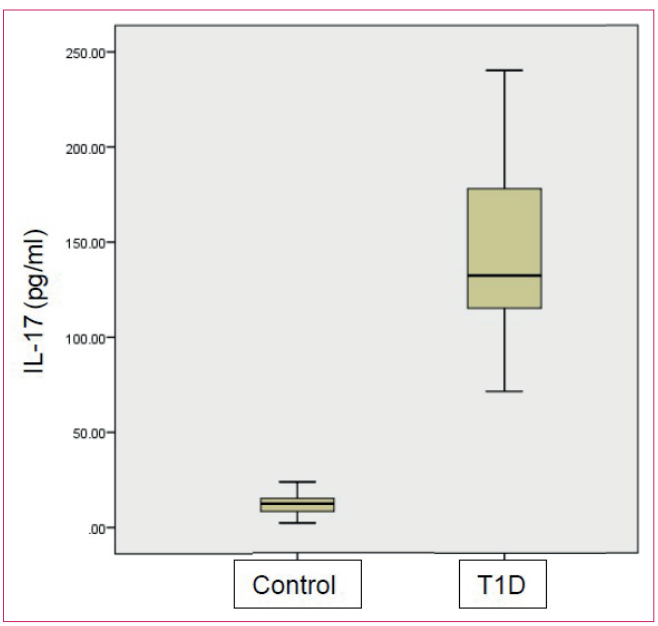

Figure 2. Mean IL-17 levels in control and patients with T1D group p<0.001

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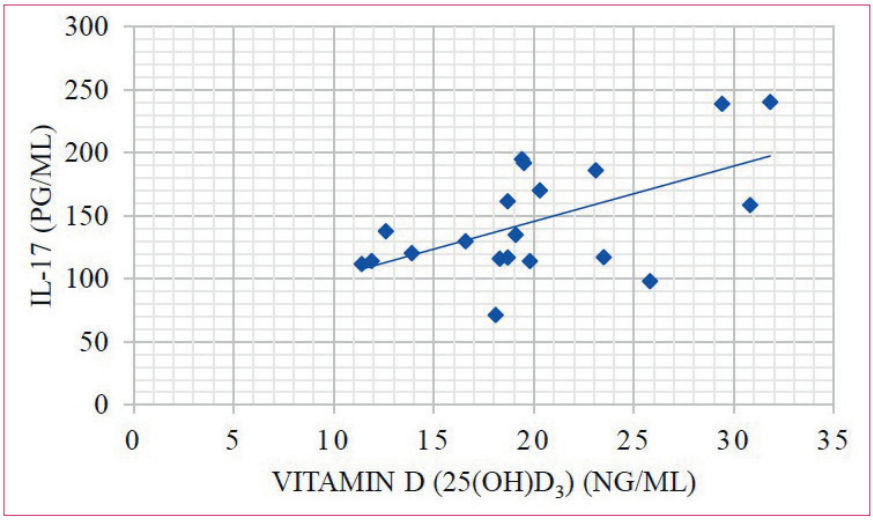

Figure 3. Correlation between serum vitamin D 25(OH)D_3 and IL-17 levels in children with T1D ($p<0.05$; $r=+0.566$)

Correlation Between Vitamin D 1.25(OH)$_2D_3$ and Interleukin-17 Serum Level in Children with T1D

Kolmogorov-Smirnov normality test revealed that the data were normally distributed ($p>0.05$). Vitamin D and IL-17 serum level in children with T1D also revealed a statistically significant positive correlation with $p<0.05$ and $r=+0.566$ (Figure 3).

Discussion

The higher level of IL-17 in the T1D group showed that immune response might play an important role in the pathogenesis of T1D. Previous studies showed a higher level of IL-17 secretion in peripheral venous blood of T1D subjects compared with healthy control group.[19] Expression of RORC2 mRNA in those studies were increased in response to T cell activation in T1D compared with healthy control group.[19] Another study showed that T1D subjects with new-onset disease (<6 month from diagnosis) had a 2.6-fold and 3.1-fold higher level of $CD4^+$ and $CD8^+$ T cells that secrete IL-17 compared to controls.[26] Additionally, a study to compare level of IL-17 in long term T1D subjects and healthy control group showed similar result. The study showed that long term T1D subjects had a moderate increase of IL-17-secreting cells.[27]

IL-17 is a pro-inflammatory cytokine that contributes to the initiation and propagation of autoimmune inflammation. There are six known isoforms of IL-17, from A to F, but only IL-17A and IL-17F are produced by Th17. These are pro-inflammatory cytokines differentiated from naïve $CD4^+$T cells that promote inflammation in autoimmune diseases through stimulation of inflammatory mediator production, as well as other immunological responses, such as imbalance between Th17, regulatory mechanisms, and Th1 response.[28–30,31] IL-17 secretion are likely to disrupt the delicate balance of islet T cells in favor of autoimmune inflammatory destruction.[26] The increased levels of IL-17 in T1D may be attributed to the presence of pro-inflammatory cytokine milieu that drives toward Th17 differentiation. Actually, monocytes from T1D patients spontaneously secrete substantially higher levels of IL-6 and IL-1β which promote IL-17 production by memory $CD4^+$ T cells.[27] Stabilization of Th17 cells is achieved through activation of STAT3 and partially STAT4 through interaction of IL-23-producing APCs.[29] Th17-related transcription factor RORC2 and IL-22 cytokine is increased in T1D with CCR6 expression.[19] CCR6-expressing T lymphocytes show specific recruitment to the mucosal surfaces in inflamed islets.[19] IL-17 enhances transcription of SOD2, and in synergy with IL-1β and IFN-γ, also enhances transcription of the inducible isoform of NOS2A and COX-2, which are involved in the inflammatory response in islet cells. IL-17 also enhances the pro-apoptotic responses in human islet cells by inhibit BCL-2 gene expression.[19,27]

The regulation of cytotoxic T lymphocytes have been indirectly modulated by active form of vitamin D (1.25(OH)$_2D_3$) which protects the β-cells from damaging immune attack.[23] Serum level of vitamin D has been implicated in the pathogenesis of type 1 diabetes mellitus in children and adolescents.[32] Pancreatic tissue, especially the β-cells express the vitamin D receptor (VDR) and variations in the genes controlling the vitamin D metabolism and expression of VDR have been implicated

in the pathogenesis of T1D.[33] Several immune cells express the VDR as vitamin D-activating enzymes. Activation of VDR signaling inhibits DC maturation which is represented by decrease DC marker, class II MHC, co-stimulator molecules (CD40, CD80, and CD86) and other maturation marker (such as CD83). DC which has been modulated by $1.25(OH)_2D_3$ will shift Th1 and Th17 dominant into Th2-dominant response and increase IL-10 secretion which leads to proliferation of suppressive Treg.[34]

Vitamin D deficiency are fairly prevalent in children and adolescents with T1D. A recent meta-analysis examining the correlation of serum vitamin D level with T1D in children found that the serum vitamin D level was significantly lower than in healthy control population.[35] In our study, we found that 60% patient with T1D had vitamin D deficiency, while 30% patient had vitamin D insufficiency, and only 10% of patients had sufficient vitamin D level. Previous cohort study showed that 14.8% children with T1D had vitamin D deficiency and 31.0% of the children had low vitamin D level.[35] The other study in UK showed that 14.8% children with T1D from the whole cohort were vitamin D deficient and 31% were insufficient.[33]

Vitamin D might inhibit inflammation through inhibition of prostaglandin synthesis and actions. Therefore, vitamin D deficiency is related with inflammation-linked vascular endothelial dysfunction. Furthermore, in the present study, patients with calcitriol supplementation showed an apparent decrease of inflammatory factors.[34] $1.25(OH)_2D_3$ directly influences insulin secretion in the pancreatic β-cells through a rise in intracellular-free calcium concentration via the nonselective calcium channel, rather than the calcium dependent inositol 1.4.5-triphosphate receptor-mediated pathway. It also exerts a stimulating effect on insulin release via protein kinase A activation, but reduces the supra-normal cyclic adenosine monophosphate (AMP) synthesis, and also provide supplementary calcium to the β cell by regulating the intracellular signaling processes involving phospholipids metabolism, protein kinase C induction, $Ca2^+$ mobilization, and $Ca2^+$ entry by $Ca2^+$ channels. Vitamin D also has a direct effect on naive $CD4^+$ T cells to enhance the development of Th2 cells in the absence of APC.[32–34]

This study showed that vitamin D level was significantly correlated with IL-17 level. IL-17 as pro-inflammatory cytokine-induced inflammation of Langerhans' islets in the pancreas marked by infiltration of T lymphocyte cause an increase in reactive oxygen species generation in acinar cells contributing to β-cell destruction.[13] While vitamin D might inhibit inflammation, but vitamin D serum level in T1D is deficient. A previous in vitro study showed that administration of $1.25(OH)_2D_3$ to human T cell CD4(+)CD25(-) decrease its pro-inflammatory cytokines production (IFNγ, IL-17 and IL-21) and induce adaptive regulatory T cell activator (CTLA-4 and FoxP3).[36] However, this study had several limitations: proxy of β-cell function such as C-peptide level and other immune response alterations were not examined.

Conclusion

Our results in this study suggest that in children with T1D, there is a significant increase of IL-17 level as a major immune alteration and vitamin D deficiency induced inflammation of Langerhans' islets in the pancreas contributing to β-cell destruction in compared to unaffected children. Emerging evidence has shown that therapeutic agents in the form of vitamin D supplementation can regulate autoimmune diabetes by targeting IL-17 or directly inhibiting Th17 cells. Further, a novel potential therapeutic strategy for T1D based on the control of IL-17 immunity and vitamin D supplementation can be developed.

Ethics Committee Approval: This study was carried out in accordance with the Declaration of Helsinki.

Informed Consent: All patients and parents signed an informed consent form before participation.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper..

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Author Contributions: Harjoedi Adji Tjahjono carried out design of the study, most of the experiment, drafted the manuscript, and derived the models. Leny Silviana Farida participated in analysed the data and revised the manuscript. All authors read and approved the final manuscript.

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