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CD4^+ CD25^+ cells in type 1 diabetic patients with other autoimmune manifestations

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ABSTRACT

The existence of multiple autoimmune disorders in diabetics may indicate underlying primary defects of immune regulation. The study aims at estimation of defects of CD4^+ CD25^+high cells among diabetic children with multiple autoimmune manifestations, and identification of disease characteristics in those children. Twenty-two cases with type 1 diabetes associated with other autoimmune diseases were recruited from the Diabetic Endocrine and Metabolic Pediatric Unit (DEMPU), Cairo University along with twenty-one normal subjects matched for age and sex as a control group. Their anthropometric measurements, diabetic profiles and glycemic control were recorded. Laboratory investigations included complete blood picture, glycosylated hemoglobin, antithyroid antibodies, celiac antibody panel and inflammatory bowel disease markers when indicated. Flow cytometric analysis of T-cell subpopulation was performed using anti-CD3, anti-CD4, anti-CD8, anti-CD25 monoclonal antibodies. Three cases revealed a proportion of CD4^+ CD25^+high below 0.1% and one case had zero counts. However, this observation did not mount to a significant statistical difference between the case and control groups neither in percentage nor absolute numbers. Significant statistical differences were observed between the case and the control groups regarding their height, weight centiles, as well as hemoglobin percentage, white cell counts and the absolute lymphocytic counts. We concluded that, derangements of CD4^+ CD25^+high cells may exist among diabetic children with multiple autoimmune manifestations indicating defects of immune controllers.

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Introduction

Diabetes mellitus (DM) is a common chronic, metabolic syndrome; which results in hyperglycemia as a cardinal biochemical feature. Type 1 diabetes is the most common type of diabetes in children and adolescents. Type 1 diabetes is caused by deficiency of insulin secretion due to pancreatic B-cell damage. Most cases of type 1 diabetes are primarily due to T-cell mediated pancreatic islet β-cell destruction, which occurs at a variable rate. There are usually serological markers of an autoimmune pathologic process, including islet cell antibodies (ICA), insulin autoantibodies (IAA), glutamic acid decarboxylase (GAD), the insulinoma-associated 2 molecule (IA-2) and zinc transporter 8 (ZnT-8) [1].

Autoimmune features were considered as associations with immunodeficiency disorders but are now viewed as an
important component of some diseases attributed to the break-down of self – tolerance or defects of immune regulators [2]. Furthermore some Primary Immune Deficiencies (PID) classifications now divide diseases according to the frequency of autoimmune features [3]. CD4\(^+\) CD25\(^+\) T cells were named regulatory T cells (T reg) and since then have been intensively characterized by many groups. It has now been well documented in a variety of models that CD4\(^+\) CD25\(^+\) play indispensable roles in the maintenance of natural self-tolerance, in averting autoimmune responses, as well as in controlling inflammatory reactions [4,5].

Type 1 diabetes is a common presenting feature in primary immune deficiency disorders affecting immune control like Immune dysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX), Autoimmune Polyendocrinopathy Candidiasis-Ectodermal dystrophy (APECED) and Common Variable Immunedeficiency (CVID) [6,7]. The autoimmune disorders are often present or can even prevail over recurrent infections when the genetic defect affects regulatory T (Treg) cells, which are the major players in maintaining peripheral tolerance [8].

Treg cell subset is impaired in IPEX syndrome; a disease caused by mutations in fork head box p3 (FOXP3) gene, the master switch for the function of Treg cells [9]. Notably, around one third of the patients, with clinical manifestation closely resembling IPEX syndrome, FOXP3 is not mutated, these patients are referred to as IPEX like [10]. The contributions of an altered Treg cell in the pathogenesis of IPEX like syndromes remain elusive [11]. Treg cell detection and quantification in humans have been limited by the fact that the main markers of their identification, CD25\(^+\) and FOXP3\(^+\) are also expressed by the activated Teff cells, which can be increased in inflammatory conditions, typically in autoimmune diseases [9,12].

The study aims at estimation of the defects of CD4\(^+\) CD25\(^+\) high cells among diabetic children with multiple autoimmune manifestations, diagnosis of underlying primary immuno-deficiency disorders and indentification of disease characteristics in those children.

**Subjects and methods**

The study protocol was approved by the Institutional Review Board and the Ethical Committee of Cairo University, Egypt and informed consents were obtained from the patients’ guardians. Twenty-two children (12 females and 10 males) with type 1 diabetes mellitus with one or more of the following features: autoimmune enteropathy, autoimmune thyroiditis, autoimmune hemolytic anemia, autoimmune hepatitis and/or alopecia. Twenty-one healthy subjects matched for age and sex were assessed as a control group with no signs or symptoms of autoimmune, chronic, inflammatory and neoplastic diseases.

Detailed history taking, clinical examination with emphasis on anthropometric parameters and glycemic control over the last year of the patients were taken. Laboratory investigations included: complete blood picture, glycosylated hemoglobin, antithyroid antibodies, Celiac antibody profile and inflammatory bowel disease profile when indicated.

Peripheral venous blood was drawn using tubes containing EDTA. Blood samples were processed within 2 h of collection.

**Monoclonal antibodies**

Phycoerythrin(PE)-conjugated monoclonal anti-CD4(Catalog number FAB3791P), Phycoerythrin(PE)-conjugated monoclonal anti-CD8 (Catalog number FAB1509P), fluorescein isothiocyanate (FITC)-conjugated anti-CD3(Catalog number FAB100F) from R&D Systems Company and phycoerythrin cyanin 5 (PE-cy5)-conjugated anti-CD25(Catalog number 555433) from BD Bioscience Company.

**Flow cytometric analysis**

Immunofluorescence staining was performed on whole blood. For each case; two test tubes were prepared; in each 50 μl of
| No | Age  | One of DM | Clinical features | Infections | No of DKA | Therapy | HbA1c% (mmol/ml) | CD4⁺/CD25⁺ high |
|----|------|-----------|------------------|------------|-----------|---------|-----------------|----------------|
| 1  | 13.4 | 6.25      | Hypothyroid HT, ST | No         | 2         | Thyroxin | 10.8%(95)       | 0.44           |
| 2  | 7.7  | 2.55      | Celiac, hypothyroid HT, ST | Hepatitis A | 1         | Thyroxin | 15%(140)        | 0.28           |
| 3  | 14.4 | 7.17      | Celiac, Euthyroid HT, ST | No         | 6         | Thyroxin | 7.4%(57)        | 0.28           |
| 4  | 12.3 | 5.23      | Euthyroid HT | Sepsis (ICU) admission | 2         | Thyroxin | 9.5%(80)        | 0.45           |
| 5  | 10.9 | 8.63      | Hypothyroid HT | RTI        | 0         | Thyroxin | 7.2%(55)        | 0.36           |
| 6  | 11   | 4.42      | Hypothyroid HT, ST | No         | 1         | Thyroxin | 8.4%(68)        | 0.19           |
| 7  | 5.4  | 4.73      | Hypothyroid HT | No         | 1         | Thyroxin | 7.1%(54)        | 0.29           |
| 8  | 11.1 | 1.27      | Celiac, Ulcerative colitis, Euthyroid HT | URTI, Pneumonia, GIT with *Entamoeba histolytica* | 0         | IS       | 8.4%(68)        | 0.07           |
| 9  | 12.1 | 0.2       | Autoimmune hepatitis, ST, (Wolcott-Rallison Syndrome) | Otitis media, Chicken Pox, Roseola infection with *Klebsiella* | 1         | IS       | 10.5%(91)       | 0.09           |
| 10 | 11.5 | 4.8       | Hypothyroid HT | No         | 0         | Thyroxin | 11.5%(102)      | 0.48           |
| 11 | 6.43 | 5.38      | Euthyroid HT | No         | 0         | Thyroxin | 6.9%(52)        | 0.88           |
| 12 | 18.8 | 2.9       | Celiac | RTI        | 10        | Thyroxin | 12.5%(113)      | 0.45           |
| 13 | 14.5 | 9.9       | Celiac, ST | RTI        | 1         | Thyroxin | 13%(119)        | 0.38           |
| 14 | 12.4 | 9.7       | Euthyroid HT Aplastic anemia, SLE | EBV, CMV Oral moniliasis Wound infection with *Klebsiella and Pseudomonas* | 0         | IS       | 5%(31)          | 0.0            |
| 15 | 10.9 | 9.2       | Euthyroid HT | No         | 0         | Thyroxin | 7.45%(58)       | 0.28           |
| 16 | 4.1  | 3         | Euthyroid HT | NO        | 0         | Thyroxin | 7.4%(57)        | 0.43           |
| 17 | 15.5 | 12.3      | Crohns disease Epiliptogenic dysfunction by EEG | RTI Otitis media | 0         | IS       | 10.2%(88)       | 1.41           |
| 18 | 9.12 | 7.9       | Euthyroid HT | NO         | 1         | Thyroxin | 9.6%(81)        | 0.07           |
| 19 | 14.15| 8         | Hypothyroid HT | NO        | 1         | Thyroxin | 8.5%(69)        | 1.15           |
| 20 | 15.2 | 9.5       | Euthyroid HT, Addison disease | NO | 1         | Asitonin H, Hydrocortisone. | 12.2%(110) | 0.28 |
| 21 | 20.8 | 1         | Hypothyroid HT, Alopecia, neutropenia | Recurrent oral, vaginal ulcers and superficial abscess | 0         | Thyroxin | 7.7%(61)        | 0.79           |
| 22 | 5.35 | 5.3       | Hepatitis? Alopecia, Autoimmune hemolytic anemia | | 0         | Thyroxin | 6.3%(57) | 0.47 |

Legend: DM: diabetes mellitus, DKA: diabetic ketoacidosis, HbA1c: glycosylated hemoglobin, HT: Hashimoto thyroiditis, RTI: respiratory tract infection, URTI: upper respiratory tract infection, GIT: gastroenteritis, IS: immunosuppressive drugs, EBV: Epstein barr virus, CMV: Cytomegalovirus, EEG: electroencephalogram, ST: short stature, ICU: intensive care unit.
whole blood was added to the appropriate amount of the monoclonal anti-bodies (5 μl).

Simultaneous staining for CD3, CD4, CD25 was done and CD3 together with CD8 in the other tube.

Background fluorescence was assessed using the appropriate isotype- and fluorochrome-matched control monoclonal antibody to determine the percentage of positive cells. Lymphocytes were gated on by their forward and side scatter properties, and CD3+CD4+CD25+ high cells were determined within the lymphocytes gate.

Antibody staining analysis was performed on Beckman Coulter XL flow cytometer FACSE.

These reagents were provided by Cairo University, there was no other source of funding during conduction of the study included.

**Statistical analysis**

Parametric quantitative data were presented by mean and standard deviation (SD) and compared by *t*-student test. Nonparametric quantitative data were presented by median and interquartile range (IQR) and compared by Mann U Whitney test. Continuous data were correlated by Pearson correlation and presented by scatter plot. Receiver Operator Characteristic (ROC) curve were constructed to assess the association between CD4+CD25+ in relation to endocrinical complication.

**Results**

The age of the patients ranged from 4.1 to 20.8 years (median 11.6). There were 10 males and 12 females. Consanguinity was positive in six patients from the case group (27.3%). The average duration of diabetes was equal to five years and seven months.

The first presentation at diagnosis of diabetes mellitus (DM) was Diabetic Ketoacidosis (DKA) in 18.2% of the patients and hyperglycemia in 81.8%. Six patients received immunosuppressive drugs and eight patients received thyroxin replacement. Most of our patients suffered acute diabetic complications such as severe hypoglycemia (31.8%) and DKA (54.6%). Regarding the hypoglycemic attacks, one patient had frequent attacks of hypoglycemia before being diagnosed as Addison disease, another patient was newly diagnosed, whereas the other patients had infrequent attacks and were often related to their activity or receiving the dose of insulin without taking the proper diet. Regarding the growth parameters there were significant statistical differences between the

| Table 3 | Comparison between case and control groups regarding the Hemoglobin, WBC’s and T cell subpopulations. |
|---------|-----------------------------------------------------------------------------------|
|         | Group (n)                          | Mean     | SD       | \( P \text{ value} \) |
|---------|------------------------------------|----------|----------|---------------------|
| Hemoglobin (g/dl) | Case (22)              | 11.491   | 1.4527   | 0.01*               |
|         | Control (21)             | 12.40    | 0.5128   |                     |
| WBC’s (x10^3/μl) | Case                   | 6.464    | 3.0288   | 0.010*              |
|         | Control                | 8.524    | 1.7815   |                     |
| Neutrophil (%) | Case                   | 44.95%   | 12.124   | 0.441               |
|         | Control                | 47.52%   | 9.250    |                     |
| ANC | Case                   | 3108.77  | 2028.515 | 0.085               |
|         | Control                | 4039.10  | 1338.911 |                     |
| Lymph (%) | Case                   | 45.59%   | 11.927   | 0.724               |
|         | Control                | 44.48%   | 8.232    |                     |
| ALC | Case                   | 2763.55  | 1071.28  | 0.004*              |
|         | Control                | 3805.52  | 1153.876 |                     |
| CD3+ % | Case                   | 63.64%   | 7.6100   | 0.348               |
|         | Control                | 60.810%  | 9.9257   |                     |
| Absolute no of CD3+ | Case              | 1730.45  | 687.840  | 0.019*              |
|         | Control                | 2300.57  | 840.763  |                     |
| CD3+CD4+ % | Case               | 35.01%   | 7.1393   | 0.360               |
|         | Control                | 37.071%  | 7.4078   |                     |
| Absolute no of CD3+CD4+ | Case          | 934.14   | 336.222  | 0.002*              |
|         | Control                | 1371.1   | 495.052  |                     |
| CD3+CD8+ % | Case               | 28.268%  | 6.7149   | 0.029*              |
|         | Control                | 23.762%  | 6.3231   |                     |
| Absolute no of CD3+CD8+ | Case          | 800.55   | 428.011  | 0.415               |
|         | Control                | 901.81   | 374.601  |                     |
| CD4+CD25+high % | Case             | 0.430%   | 0.34913  | 0.82                |
|         | Control                | 0.4086%  | 0.2643   |                     |
| MFI | Case                   | 0.55     | 1.342    | 0.069               |
|         | Control                | 2.93     | 5.834    |                     |
| Absolute no of CD4+CD25+high | Case   | 3.0412   | 3.73     | 0.099               |
|         | Control                | 4.2380   | 5.55     |                     |

WBC: white blood cells, ANC: absolute neutrophilic count.

The absolute count of CD4+CD25+ was done by Mann Whitney U test (Median, IQR), the others were done by *T*-test.

\( P < 0.05 \).
case and control groups regarding their height, weight according to the Egyptian growth chart, with P-value of 0.004, 0.05 respectively as shown in Table 1.

Seven patients were short in stature (below the 3rd percentile for age and sex) as shown in Fig. 1. Cases numbers (2, 3) were diagnosed as type 1 DM, autoimmune thyroid disease and Celiac disease. Cases numbers (1, 6) were diagnosed as type 1 DM and hypothyroid Hashimoto’s thyroiditis. Case number (9) was diagnosed as type 1 DM and Wolloct–Rallison Syndrome. Case number (13) was diagnosed as type 1 DM and Celiac disease. Case number (22) was diagnosed as type 1 DM and autoimmune hemolytic anemia with alopecia. Six patients also had delayed pubertal stages for their age cases no (1, 3, 4, 8, 9 and 13).

The study group had poor glycemic control. Five patients had glycosylated hemoglobin levels HbA1c >8.5% (69 mmol/mol), while thirteen cases had HbA1c >8% (64 mmol/mol), with duration of diabetes 5.73 years, SDS (5.08), putting them at risk of the chronic complications of diabetes. Six of these patients (27.3%) had renal complication in the form of persistent microalbuminuria or slight impairment of the renal function, five of them had a duration of diabetes >5 years cases no (1, 2, 8, 9 and 20) and only one patient with 3.2 years duration case no (14). The renal affection of the former patient cannot be contributed to diabetes alone as this patient had multiple autoimmune phenomena and was diagnosed as Systemic Lupus Erythematosus (SLE). Two patients (9.1%) suffered from neuropathy and one patient (4.5%) from arthropathy.

The most frequent clinical autoimmune feature associated with type 1 diabetes in the cases was endocrinopathy (77.2%) in the form of Hashimoto’s thyroiditis with positive antithyroid antibodies followed by enteropathy in 27.2% of the cases. The celiac patients represented 22.7% of the total patients whereas inflammatory bowel disease constituted 9% (one patient had Celiac and Ulcerative colitis diseases, case no 8). There was one case with autoimmune hepatitis, one case with autoimmune hemolytic anemia and one case diagnosed as Systemic Lupus Erythematosus (SLE) as demonstrated in Table 2.

Regarding the blood counts in the case group, there were three patients with leucopenia with white blood cell counts (WBC) ≤ 4 × 10^9/l (cases # 2, 14, 17). There were five patients with neutropenia with absolute neutrophilic counts (ANC) < 1500, case no 1(1290), case no 2(880), case no 14 (575), case no 17(816) and case no 21 (1300). There were two patients with lymphopenia case no 8 with an absolute lymphocytic count (ALC) of (1222) and case no 17 (1344). Significant statistical differences were observed between case and control groups regarding hemoglobin percentage, WBC’s and the

Fig. 2 Comparison of the absolute number of CD3^+ CD4^+ in cases and control group.

Fig. 3 Flow cytometric results of patient 14: The lymphocyte, as it was identified by their forward and side scatter properties were gated for coexpression of CD4^+ and CD25^+high. CD4^+CD25^+high = 0%.
absolute lymphocytic counts with \( P \)-values of 0.01, 0.01, 0.004, respectively as shown in Table 3.

As for CD Counts, there were statistically significant differences regarding the absolute CD3\(^+\) count, the absolute CD4\(^+\) counts and the CD8\(^+\) percentage results between the case and control groups, with \( P \)-values of 0.019, 0.002 and 0.02 respectively as seen in Table 3 and Fig. 2.

Four cases showed CD4\(^+\)CD25\(^+\)high percent less than 0.1\%, (cases numbers 8, 9, 14 and 18), and their clinical features as well as infection histories were described in Table 2. The lower percentage of CD4\(^+\)CD25\(^+\)high was a continuous not transient event, in case no (9) immunosuppressive treatment was stopped five years ago before the study while in case no (18) immunosuppressive drugs were never received, as for case no (8) she was on Azathioprine and Pentaza during the study and regarding case no (14) he was on pulse steroid therapy, Sandimmune and Cellcept. The flow cytometry results of patient number 14 show CD4\(^+\)CD25\(^+\)\% = 0\% in Fig. 3.

There was no statistically significant difference between the two studied groups regarding the percentages or the absolute number of CD4\(^+\)CD25\(^+\)high by analysis of Roc curve as in Fig. 4 and Fig. 5.

Also there was no statistically significant difference between mean fluorescence intensity MFI in patients when compared to the healthy group (See Fig. 6).

**Discussion**

The consanguinity rate in the diabetic group was (27.3\%), higher rates were reported in Saudi diabetic children [13]. In another study investigating cases with CD25\(^+\) deficiency, only two male patients were described; one of them from a positive consanguineous family [14].

In our study group, four patients (18.2\%) presented by DKA as a first manifestation of T1DM, this frequency is limited to our group only, as it is lower than the frequency of DKA being a first presentation in Diabetic Endocrine and Metabolic Pediatric Unit (DEMPU), which receives 30–50 newly diagnosed type1 diabetic patients monthly, with 30–40\% of them presenting with DKA (personal communication) as well as other studies with a range of 26.3–55.3\% [15,16].

Formerly, Type1 DM was known to have adverse effects on linear growth and pubertal development [17]. However, with recent insulin treatment regimens and monitoring of blood glucose level, growth has substantially improved and height in children with TIDM today should be similar in all ages to the height of their unaffected peers [18].

The significant statistical differences between the case and the control groups regarding their height and weight point to the multifactorial influence of their disease conditions, associations and treatment regimens. Five patients of them had glycosylated hemoglobin levels (HbA1c) more than 8.5\%, while 13 cases had HbA1c of more than 8\% indicating poor control. These results are in concordance with Danne et al. who showed a direct correlation between increased glycosylated hemoglobin levels and standing height SDS reduction [19] and Gunczler et al. who also showed that children with poor control have a significantly lower growth velocity compared with well controlled subjects [20].
On the other hand, the development of chronic complications in diabetes is related to the hyperglycemia that persists even with treatment of the disease, it is also dependent on the duration of diabetes [21].

Our results showed that diabetic nephropathy was the most common complication among this cohort of diabetic patients, in concordance with other studies [22]. In contrast other studies reported a different incidence, where retinopathy was the most common complication followed by neuropathy and nephropathy [13].

Two patients (9.1%) had neuropathy, one patient with duration of diabetes of 19.2 years case no (21) and the other patient case no (14) was diagnosed as autoimmune polyneuropathy.

Several autoimmune features were detected in some cases necessitating vigilance to pick up those problems that may present in a subtle form in diabetics. Thyroid autoantibodies ranked first as the most commonly associated autoimmune disorder among diabetic patients in concordance with studies that estimate percentage ranging from 11% to 46% of diabetic patients with either thyroid peroxidase antibodies or thyroglobulin antibodies [23]. The prevalence of Celiac disease in patients with diabetes ranges from 4.4% to 11.1% compared to the general population [24,25]. In our study group the celiac patients represented 22.7% of the total patients whereas inflammatory bowel disease constituted 9% of the study group.

There was a significant statistical difference between cases and controls groups in hemoglobin percentage and white blood counts with $P$-values (0.01, 0.01 respectively). These results are similar to those observed in laboratory abnormalities in IPEX and IPEX like syndrome where cytopenia (anemia, leucopenia, and thrombocytopenia) may be present [6,26].

Fig. 6  Flow cytometric results of patient 9: The lymphocyte, as it was identified by their forward and side scatter properties were gated (a) for co expression of CD4$^+$ and CD25$^{high}$. CD4$^+$ CD25$^{high}$ = 0.09% (b) compared to one of the healthy controls (c and d) where the CD4$^+$ CD25$^{high}$ = 1.37%.
Regarding CD4+ CD25+high proportions, there were four patients with values of less than 0.1% however values did not mount to a statistical difference between the absolute numbers of CD4+ CD25+high cells between the control and cases groups. These results were in concordance with Lindely et al. and Putnam et al., who reported that there was no significant difference in the percentage of CD4+ CD25+high between patients and healthy subjects as well as in the level of CD4+CD25+high expression per cell, when expressed as the mean fluorescence intensity [27,28] and are against Luczynski et al. who found significant statistical difference between newly diagnosed type 1 diabetes patients and normal children as regards CD4+CD25+ % but not the absolute counts **[29].

One explanation may be due to CD4+ CD25+ expression by the activated T effectors cell which can be increased in inflammatory and autoimmune diseases [12]. Another explanation is that Treg may demonstrate reduced functional capacity with drop of CD4+ CD25+ levels over time [30].

Other studies indicate the defect may involve the number and/or function of Tregs in type 1 DM [31]. Barzaghi et al., reported that CD4+ CD25+ FOXP3+ T cells median values obtained in IPEX-like patients were not significantly lower than those detected in healthy controls, but by using demethylation analysis of FOXP3 locus; results showed quantitative defect of regulatory T cells in patients than healthy control with statistical significance difference [11].

In case no 14, the patient was diagnosed as SLE while his CD4+ CD25+ % was zero. This patient suffered from polyneuropathy followed by diabetes then two years later he developed pancytopenia, with positive Anti nuclear Antibodies (ANA), Anti double stranded Antibodies (Anti DNA), and developed rapid renal affection with lupus cerebritis. He also had positive anti-thyroglobulin antibodies and antimicrosomal antibodies with normal thyroid function. This finding was similar to most of studies that found a significant decreased percentage of CD4+ CD25+ cells in patients with SLE as compared to healthy controls [32–34].

Other studies showed that patients who were untreated and/or newly diagnosed with SLE, showed negative correlation between percentage of CD4+CD25+ and the clinical activity of the disease, this was also noted with pediatric patients and some studies reported an inverse correlation between number of CD4+ CD25+ and some studies reported an inverse correlation be-

Another explanation is the activated T effectors cell which can be increased in inflam-

matory and autoimmune diseases [12]. Another explanation is that Treg may demonstrate reduced functional capacity with drop of CD4+ CD25+ levels over time [30].

In conclusion, diabetic children with multiple autoimmune features may demonstrate CD4+ CD25+high cells deficiency favoring the immune disequilibrium.

Conflict of interest
The authors have declared no conflict of interest.

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