

CD3⁺CD56⁺ T Lymphocytes Are Associated With ER Stress and Inflammasome Activation in Type 1 Diabetes

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Abstract. *Background/Aim:* The T cell's flexibility of the immune system to be regulated affects the onset of type 1 diabetes (T1D). However, the mechanisms of endoplasmic reticulum (ER) stress and inflammasome activation in the circulating CD3⁺CD56⁺ T cells of patients with T1D remain unclear. This study evaluated the role of CD3⁺CD56⁺ T cells in T1D and their correlations with ER stress, inflammasome activation and disease characteristics. *Materials and Methods:* The frequency of circulating CD3⁺CD56⁺ T cells was determined using flow cytometry in healthy individuals and patients with T1D. Calnexin, NLR family pyrin domain containing 3 (NLRP3), ASC, caspase-1 (Casp1), cleaved caspase-3 (C-Casp3), and annexin V (AnnV) expression and propidium iodide staining in CD3⁺/CD56⁺ T cells were analyzed using flow cytometry. *Results:* The frequency of

CD3⁺CD56⁺ T cells was reduced in patients with T1D relative to that in healthy individuals. In addition, high calnexin, NLRP3, ASC and Casp1 expression in CD3⁺CD56⁺ T cells was negatively correlated with the percentage of CD3⁺CD56⁺ T cells in patients with T1D. *Conclusion:* ER stress, inflammasome activation, and a lower peripheral frequency of circulating CD3⁺CD56⁺ T cells might indicate disease progression and necessitate clinical T1D immunological self-tolerance monitoring.

Type 1 diabetes (T1D) is an autoimmune disorder that develops when insulin-producing β -cells in the islets of Langerhans are destroyed in an organ-specific manner (1, 2). Bidirectional interactions between immune cells and β -cells, such as the development of β -cell autoantibodies and the immunological assault on β -cells by CD4⁺ T helper cells, CD8⁺ cytotoxic T cells and B cells, define this complicated illness (3, 4). The immunological death of β -cells leads to decreased glucose absorption in peripheral tissues, increased hepatic glucose synthesis and hyperglycemia, all of which have catastrophic consequences on metabolic health and lead to severe complications such as retinopathy, heart disease and kidney disease. However, the pathogenesis of this disease remains unclear (1, 5, 6).

A subset of T cells co-expressing CD3 and CD56 (CD3⁺CD56⁺ T cells) has been found in the peripheral blood of healthy people and linked to several autoimmune diseases (7-10). The frequency of CD3⁺CD56⁺ T lymphocytes was lower in the peripheral blood of seven pairs of T1D-discordant monozygotic twins and triplets (11, 12). Reduced β -cell activity and the prevalence of diabetic ketoacidosis are

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linked to a low frequency of circulating CD3⁺CD56⁺ T cells (12, 13). Furthermore, CD3⁺CD56⁺ T cells from diabetic twins did not produce interleukin (IL)-4 following T cell receptor treatment, implying functional abnormalities in the surviving CD3⁺CD56⁺ T cell population in patients with diabetes (12). CD3⁺CD56⁺ T cells can decrease CD8⁺ T cell activity *in vitro* by lowering the levels of intracellular reactive oxygen species, which are essential because autoreactive CD8⁺ T cells damage insulin-producing β -cells (13). The treatment of diabetes in non-obese diabetic (NOD) mice *via* an infusion of CD3⁺CD56⁺ T cell-enriched thymocyte preparations revealed that CD3⁺CD56⁺ T cells might participate in the regulation of islet cells (14, 15).

The endoplasmic reticulum (ER) is a network of linked organelles with a variety of morphologies that spreads promiscuously across the cytoplasm, maintains cellular homeostasis and forms numerous connections with other organelles (16). Excessive ER demand by increased necessity secretion of the protein, chronic inflammation and environmental stressors induce ER stress, and following the adaptive unfolded protein response (UPR), a response is initiated to restore cellular stability (16, 17). Chronic ER stress causes the UPR to flip from pro-survival to pro-apoptosis, leading to cell death (18). The development of inflammatory and autoimmune illness has been linked to ER stress and a malfunctioning UPR (16). The NLR family pyrin domain containing 3 (NLRP3) inflammasome can be triggered by ER stress, and caspase 1 (Casp1) activation can increase the maturation and release of IL-1 and IL-18 (19). However, the involvement of ER stress and inflammasome activation in CD3⁺CD56⁺ T cell dysregulation, as well as the start and propagation of autoimmune responses in T1D, are unknown.

This study demonstrated that ER stress and inflammasome activation are essential for the dysfunction of CD3⁺CD56⁺ T cells during T1D pathogenesis and clarified their relationship with clinical characteristics.

Materials and Methods

Clinical participants. The Global International Diabetes Federation and the International Society for Pediatric and Adolescent Diabetes Guidelines for Diabetes in Childhood and Adolescence guidelines were used to diagnose T1D. The following were the inclusion criteria for patients with T1D, and whenever two of the next three prerequisites are satisfied: 1) diabetic ketosis or ketoacidosis within 3 months after the appearance of hyperglycemic symptoms; 2) a need for ongoing insulin therapy following a diabetes mellitus diagnosis and 3) a positive anti-islet autoantibody test result or the presence of endogenous insulin insufficiency without verified anti-islet autoantibodies. The exclusion criteria were as follows: 1) myocardial infarction, coronary artery bypass surgery or coronary angioplasty within the preceding 8 weeks before recruitment; 2) impaired hepatic function; 3) HIV and hepatitis B/C infection; 4) participation in any other ongoing clinical trial; 5) any other life-

threatening non-cardiac condition; 6) use of an investigational medicine or treatment regimen within 30 days following study completion; 7) use of any diabetic medication other than insulin (metformin, pramlintide or thiazolidinediones); 8) use of steroids or other immune modulators; 9) pancreatitis, gastroparesis, end-stage renal disease and medullary thyroid cancer history; 10) pregnancy; 11) obesity (BMI >30 kg/m²); 12) smoking; 13) vaccinations or infections within 8 weeks before recruitment and 14) personal or family history of autoimmune diseases.

At China Medical University Children's Hospital, participants with newly diagnosed T1D and those diagnosed 5 years previously were recruited. Healthy individuals with a fasting blood glucose level of <100 mg/dl matched with patients with T1D regarding sex, age and BMI were also recruited. At least two anti-islet autoantibodies were detected in all patients with T1D. The study was conducted according to the Declaration of Helsinki and approved by the China Medical University Children's Hospital institutional review board and the Ethics Committee (CMUH-103-REC2-046). A documented informed consent form was signed by all adult participants, as well as the parents of children who participated.

Blood samples from patients with T1D and healthy individuals were collected and processed in BD ACD Vacutainers and processed within 4 h of collection. Serum or plasma was obtained after centrifugation and stored at 80°C until use. The remaining blood samples were processed, and peripheral blood mononuclear cells (PBMCs) were collected using Ficoll-Paque (GE Healthcare Life Sciences, Taichung, Taiwan) gradient centrifugation.

Flow cytometry and cell isolation. Flow cytometric cell sorting (FACSria III cell sorter; BD Biosciences, Taichung, Taiwan, ROC; $\geq 95\%$ purity) was used to purify CD3⁺CD56⁺ T cells from PBMCs. The cells were grown in full RPMI-1640 medium with 10% heat-inactivated human AB serum (Sigma-Aldrich, Taichung, Taiwan, ROC). Surface markers on CD3⁺CD56⁺ T cells were assessed using multiparametric flow cytometry and the following antibodies: APC mouse anti-human CD3 (BioLegend, Taichung, Taiwan, ROC; clone UCHT1), PE mouse anti-human CD4 (BioLegend; clone RPA-T4) and FITC mouse anti-human CD56 (BioLegend; clone MEM-188).

Calnexin, NLRP3, ASC, Casp1 and cleaved caspase-3 (C-Casp3) staining and flow cytometry. The Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Taichung, Taiwan, ROC, catalogue no. 00-5523-00) was employed for cell fixation (2×10^6 cells/ml) and permeabilization. Before incubation, the cells were washed twice in PBS before being blocked with 1% BSA for 30 min at room temperature, followed by incubation with antibodies against calnexin (1:200, Cell Signalling, Taipei, Taiwan, ROC), NLRP3 (1:200, Cell Signalling), ASC (1:200, Santa Cruz, Taipei, Taiwan), Casp1 (1:200, Santa Cruz) and C-Casp3 (1:200, Cell Signalling) for 30 min at 4°C. After three washes with PBS, cells were incubated with fluorescence-conjugated Alexa Fluor 488 (1:200, Sigma-Aldrich) and Alexa Fluor 647 anti-rabbit secondary antibodies (1:200, Abcam, Taipei, Taiwan, ROC) for 45 min at room temperature. After incubation in 400 μ l of 1 \times binding buffer that was gently mixed with the cell solution, the cells were washed twice in PBS. A flow cytometer (Beckman, Taipei, Taiwan, ROC, CytoFlex) and APC channels were used to measure target protein expression. Using CytExpert software (Beckman), the percentages of cells positive for various dyes were calculated and examined.

Annexin V (AnnV)/propidium iodide (PI) staining in CD3⁺CD56⁺ T cells. To evaluate cell death, CD3⁺CD56⁺ T cells were stained with FITC-conjugated AnnV (Strong Biotech Corporation, Taichung, Taiwan, ROC) and PI (Strong Biotech Corporation), and AnnV buffer (Strong Biotech Corporation) was used to stain the cell suspension according to the manufacturer's instructions. RPMI-1640 medium was used to culture CD3⁺CD56⁺ T cells (1×10⁶ cells/ml) into 24-well plates. To detect cell death and apoptosis, cells were washed in PBS and stained with AnnV and PI using the Annexin V-FITC Apoptosis Detection Kit (Strong Biotech Corporation) according to the manufacturer's instructions. The cells were washed twice and diluted with 1× binding buffer. Then, 2 µl of Alexa Fluor 488-AnnV and 2 µl of PI solution were added to 100-µl cell suspensions and incubated for 15 min at room temperature. After the incubation, 400 µl of 1× binding buffer were gently mixed with the cell suspension and quantified using a flow cytometer in the FITC (AnnV) and PE (PI) channels (Beckman, CytoFlex). Using CytExpert software, the percentages of cells positive for various dyes were calculated and examined.

Statistical analysis. Prism 9 was used for statistical analysis and data visualisation (GraphPad, San Diego, CA, USA). When the sample size per group was >3, the Mann-Whitney test was employed to compare data between two groups. If the sample size per group was less than three, Student's *t*-test was performed. For comparisons among three groups, one-way analysis of variance was performed, and pairwise comparisons were performed using Tukey's method. We used Pearson's correlation coefficient to determine the associations of NLRP3, Casp1 and ASC protein levels in CD3⁺CD56⁺ T cells. All data are shown as the mean±SEM. *p*<0.05 indicated statistical significance, and statistical parameters are listed in each figure legend.

Results

Baseline demographic characteristics of patients with T1D. Table I shows the demographic characteristics of patients with T1D and healthy donors (HDs). The patients with T1D had an average age of 18.42±6.85 years and a BMI of 21.98±4.71 kg/m², and these clinical data were matched with the HDs (average age of 20.13±1.94 years, *p*=0.18 and BMI of 21.03±2.56 kg/m², *p*=0.34, Table I) to exclude the effects of sex, age, and BMI. There were significant differences in the baseline characteristics between HDs and patients with T1D for HbA1c (5.23±0.28% vs. 9.43±2.46%, respectively; *p*<0.001), Total cholesterol (157.53±23.44 mg/dl vs. 195.97±40.01 mg/dl, respectively; *p*<0.001), high density lipoprotein (58.08±10.41 mg/dl vs. 69.43±19.63 mg/dl, respectively; *p*=0.007), low density lipoprotein (91.09±23.46 mg/dl vs. 110.84±31.85 mg/dl, respectively; *p*=0.008), and triglycerides (68.47±30.26 mg/dl vs. 119.54±125.73 mg/dl, respectively; *p*=0.03). Patients with T1D have an HbA1c value of more than 8%, indicating that their diabetes is poorly managed.

Patients with T1D exhibited significantly lower CD3⁺CD56⁺ T lymphocyte counts in peripheral blood. To understand the pathophysiological significance of CD3⁺CD56⁺ T cells and

Table I. Clinical characteristics of healthy donors and patients with T1D.

Parameters	HDs	T1Ds	<i>p</i> -Value
	(n=30) Mean±SD	(n=30) Mean±SD	
Sex (male/female)	18/12	20/10	
Age (year)	20.13±0.94	18.42±6.85	0.18
BMI (kg/m ²)	21.03±2.56	21.98±4.71	0.34
HbA1c (%)	5.23±0.28	9.43±2.46	<0.001
Diabetes duration (year)		8.67±5.89	
Total cholesterol (mg/dl)	157.53±23.44	195.97±40.01	<0.001
HDL (mg/dl)	58.08±10.41	69.43±19.63	0.007
LDL (mg/dl)	91.09±23.46	110.84±31.85	0.008
Triglyceride (mg/dl)	68.47±30.26	119.54±125.73	0.03

HDL: High density lipoprotein; LDL: low density lipoprotein.

their potential correlation with disease onset, we compared circulating CD4⁺ and CD3⁺CD56⁺ T cell counts between patients with T1D (n=30) and healthy individuals (n=30, Table I). The gating strategy utilized to analyze CD4⁺ and CD3⁺CD56⁺ T cells in PBMCs from healthy individuals and patients with T1D patients is presented in Figure 1A. Compared to the findings in healthy individuals, individuals with T1D exhibited significantly lower circulating CD4⁺ (Percentage in parent, 32.13±1.39 vs. 23.62±0.27, respectively; *p*=0.002, Figure 1B) and CD3⁺CD56⁺ T cell counts (Percentage in parent, 1.99±0.27 vs. 0.96±0.14, respectively; *p*=0.002, Figure 1C) in peripheral blood.

ER expansion in circulating CD3⁺CD56⁺ T cells of patients with T1D. The inflammatory signals of T1D influence ER biogenesis and expansion to a certain extent. Consequently, we investigated whether the ER expansion phenotype was present in CD3⁺CD56⁺ T cells from patients with T1D. By labelling calnexin (an ER marker) in CD3⁺CD56⁺ T cells from healthy individuals and patients with T1D and calculating the calnexin median fluorescence intensity (MFI), we confirmed the expansion of the ER. The fluorescence intensity of calnexin exhibited a significant shift in patients with T1D compared to that in healthy controls (Figure 2A). The calnexin signature was increased in CD3⁺CD56⁺ T cells from T1D patients (calnexin MFI, 10,191±2,799 vs. 26,198±9,065, respectively; *p*=0.002, Figure 2B).

Inflammasome activation in the circulating CD3⁺CD56⁺ T cells of patients with T1D. Inflammasome activation is induced to a certain degree by ER expansion-related signals. To confirm the specific involvement of the NLRP3 inflammasome, we analysed NLRP3 (NLRP3 MFI, 7,710±630 vs. 14,808±1,206, respectively; *p*<0.001, Figure 3A), ASC (ASC MFI, 93,214±14,720 vs. 480,500±75,767, respectively; *p*<0.001,

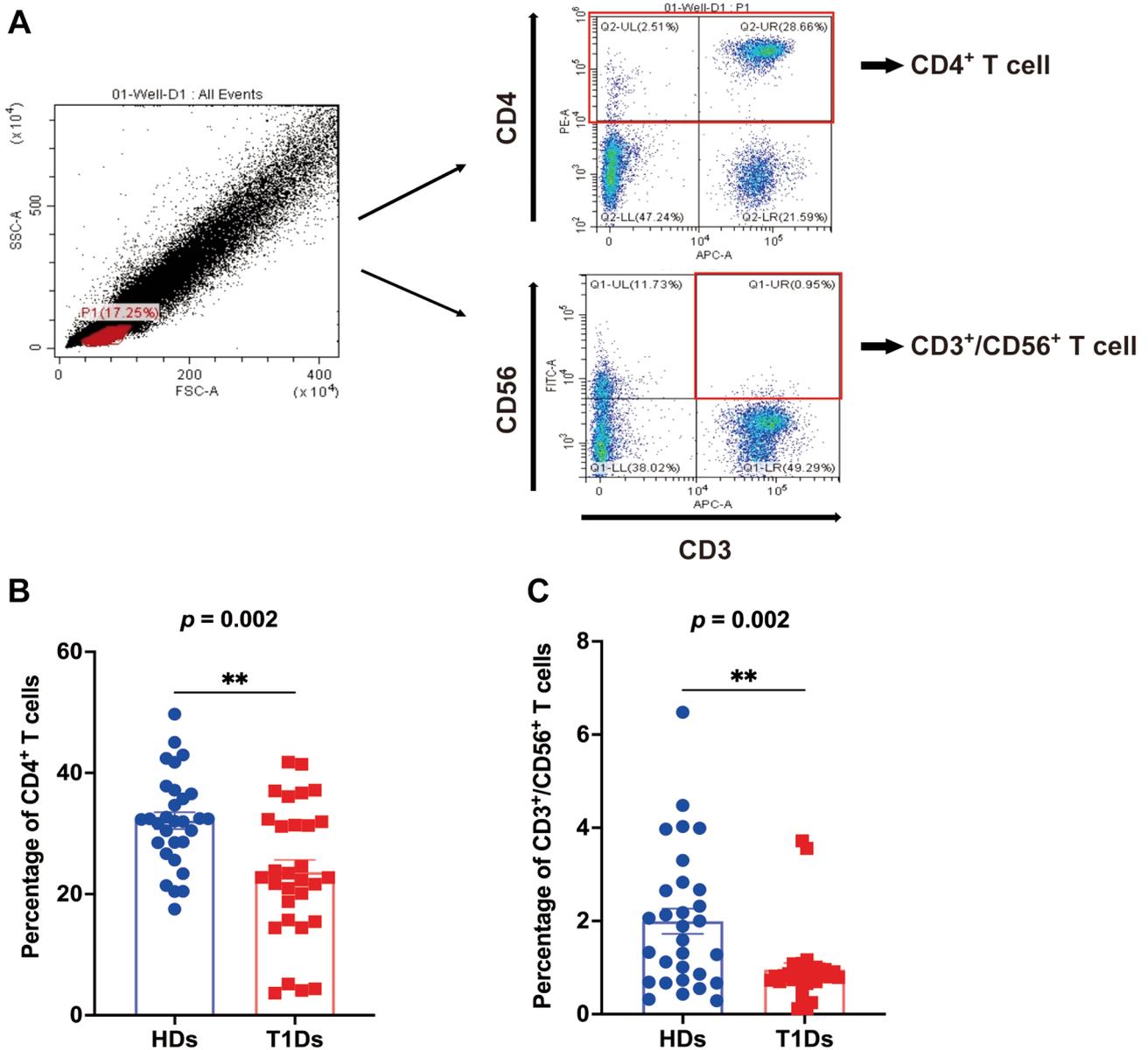


Figure 1. Peripheral blood mononuclear cells (PBMCs) from patients with type 1 diabetes (T1D) had a significantly smaller CD3⁺CD56⁺ T cell population. (A) The expression of CD3⁺ and CD56⁺ on T cells from healthy individuals and patients with T1D was assessed using a representative gating strategy of flow cytometry plots. (B) The forward scatter/side scatter (FSC/SSC) distribution and the gate (region P1) used to select cells for analysis are presented in the dot plot. (C) The proportions of CD4⁺ and CD3⁺CD56⁺ T cells among lymphocytes from healthy individuals (n=30) and patients with T1D (n=30). Values are given as the mean±SEM. Data were analysed using the two-tailed unpaired Mann-Whitney test (B-C): * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 3B) and Casp1 (Casp1 MFI, 71,157±11,053 vs. 167,366±26,247, respectively; $p < 0.001$, Figure 3C) expression in CD3⁺CD56⁺ T cells from healthy individuals and patients with T1D using a flow cytometer. We found that NLRP3, ASC and Casp1 expression was markedly increased in the CD3⁺CD56⁺ T cells of patients with T1D (Figure 3A-C).

Correlation of ER expansion and inflammasome markers with the percentage of CD3⁺CD56⁺ T cells in patients with T1D. We next measured the correlation of ER expansion and inflammasome markers with the percentage of CD3⁺CD56⁺ T cells in patients with T1D. Pearson's correlation analysis revealed a negative correlation between calnexin expression

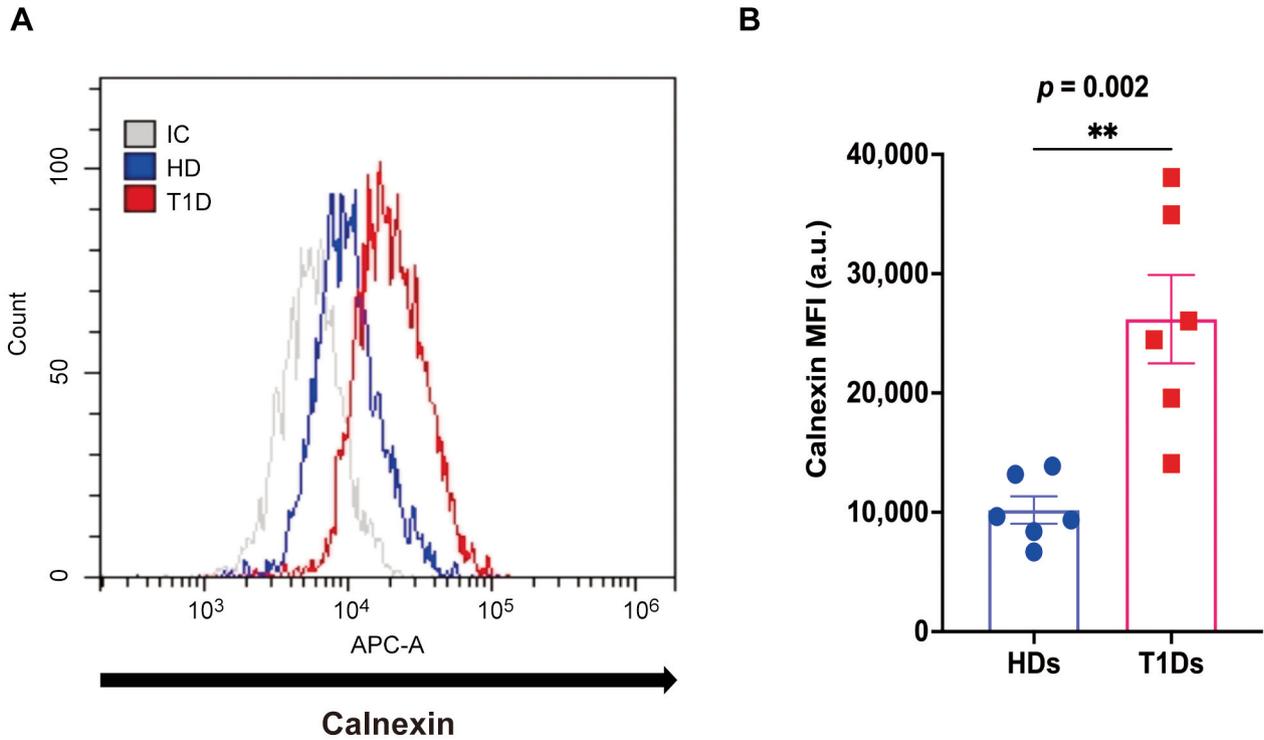


Figure 2. Endoplasmic reticulum (ER) expansion in circulating CD3⁺CD56⁺ T cells from patients with type 1 diabetes (T1D). (A) Expanded ER size in circulating CD3⁺CD56⁺ T cells from patients with T1D. Calnexin (ER marker) staining histogram and collective median fluorescence intensity (age-, sex- and BMI-matched healthy individuals, n=6; patients with poorly controlled T1D, n=6). Values are given as the mean±SEM. Data were analysed using the two-tailed unpaired Mann-Whitney test (B): *p<0.05, **p<0.01 and ***p<0.001.

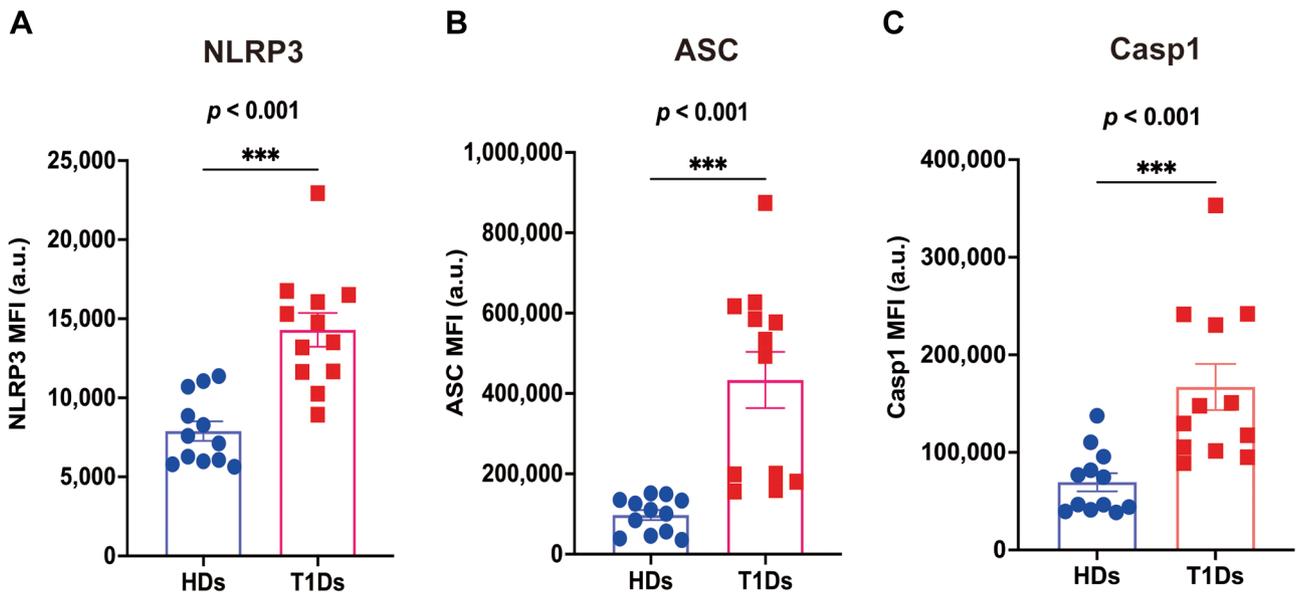


Figure 3. Inflammasome activation in circulating CD3⁺CD56⁺ T cells from patients with type 1 diabetes (T1D). (A) NLR family pyrin domain containing 3, (B) ASC and (C) caspase 1 expression and collective median fluorescence intensity in circulating CD3⁺CD56⁺ T cells from patients with T1D (age-, sex- and BMI-matched healthy individuals, n=12; patients with poorly controlled T1D, n=12). Values are given as the mean±SEM. Data were analyzed using the two-tailed unpaired Mann-Whitney test (A-C): *p<0.05, **p<0.01 and ***p<0.001.

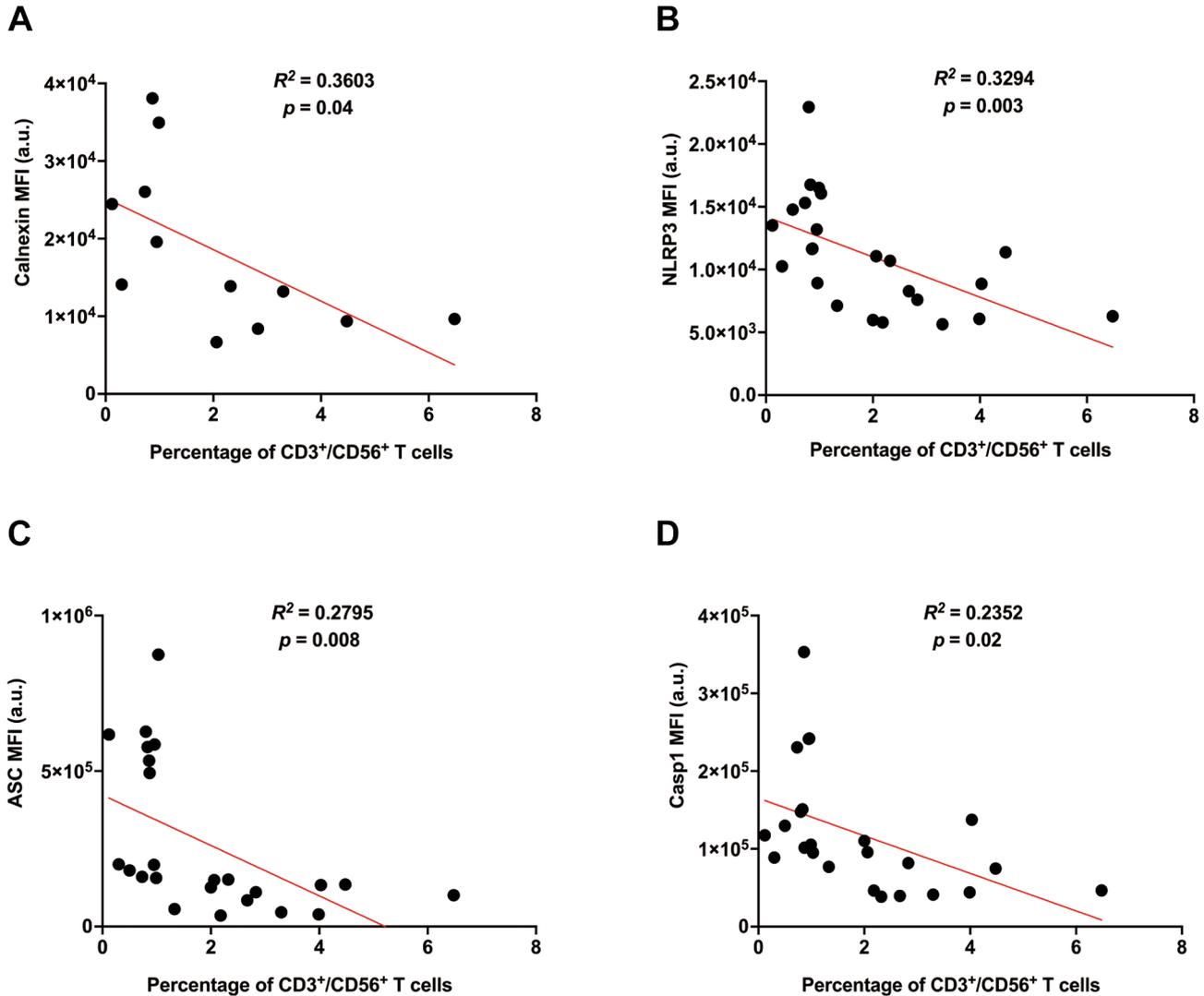


Figure 4. Correlations between inflammasome markers and the percentage of CD3⁺CD56⁺ T cells in patients with type 1 diabetes (T1D). Relationships of (A) calnexin (n=12), (B) NLR family pyrin domain containing 3 (n=24), (C) ASC (n=24) and (D) caspase 1 expression (n=24) with the proportion of CD3⁺CD56⁺ T cells in healthy individuals and patients with T1D. Data were analysed using the two-tailed Pearson's correlation coefficient (A-D): *p<0.05, **p<0.01 and ***p<0.001.

(ER expansion) and the percentage of CD3⁺CD56⁺ T cells (r=0.3603, p=0.04, Figure 4A). We also compared the correlations of NLRP3, ASC and Casp1 expression with CD3⁺CD56⁺ T cell counts in T1D. The results revealed negative correlation of NLRP3 (r=0.3294, p=0.003, Figure 4B), ASC (r=0.2795, p=0.008, Figure 4C) and Casp1 expression (r=0.2352, p=0.02, Figure 4D) with CD3⁺CD56⁺ T cell counts in T1D. These results revealed the correlation of ER expansion and inflammasome markers with the percentage of CD3⁺CD56⁺ T cells in patients with T1D.

Apoptosis activation in circulating CD3⁺CD56⁺ T cells from patients with T1D. We next examined whether the decrease

in CD3⁺CD56⁺ T cell counts is attributed to susceptibility to apoptosis. We observed increased AnnV (3.29±0.60% vs. 20.30±2.93%, respectively; p<0.001, Figure 5A) and PI (0.39±0.14% vs. 1.21±0.24%, respectively; p=0.006, Figure 5B) staining in CD3⁺CD56⁺ T cells from patients with T1D compared to healthy individuals, indicating that these cells have a higher frequency of apoptosis in patients with T1D. C-Casp3 is a crucial apoptosis executor because it is wholly or partially responsible for the proteolytic cleavage of numerous essential proteins. C-Casp3 expression in CD3⁺CD56⁺ T cells was also higher in patients with T1D than in healthy individuals (7.21±1.94% vs. 18.74±2.32%, respectively; p=0.001, Figure 5C). Thus, the decreased

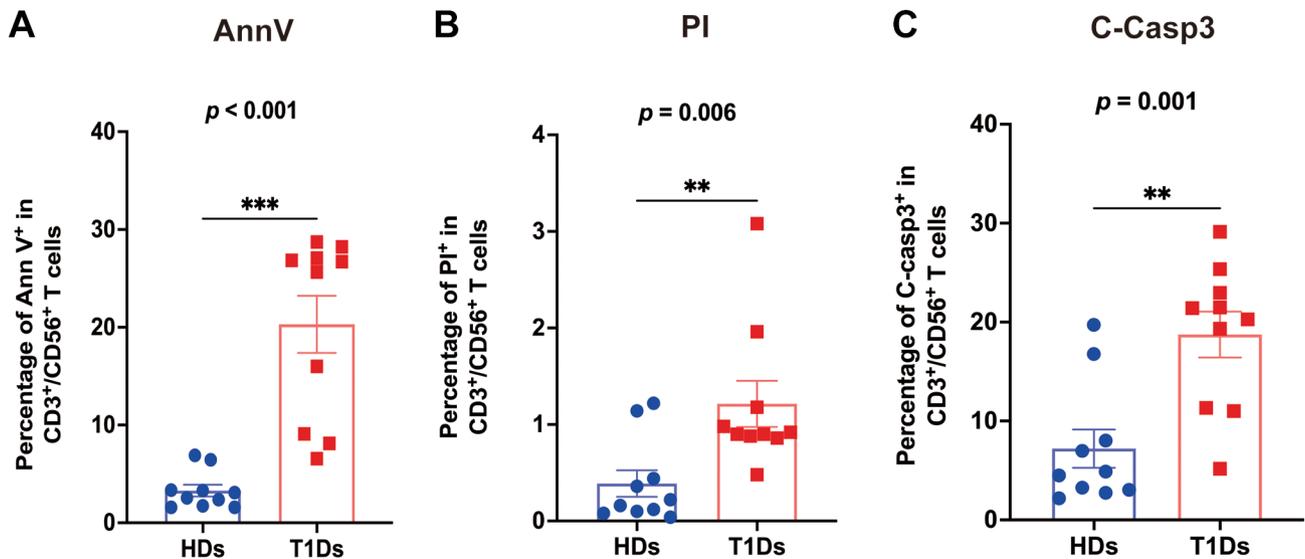


Figure 5. Increased apoptotic function in circulating CD3⁺CD56⁺ T cells from patients with type 1 diabetes (T1D). Relationship of (A) annexin V, (B) propidium iodide and (C) cleaved caspase 3 staining in circulating CD3⁺CD56⁺ T cells from patients with T1D (age-, sex- and BMI-matched healthy individuals, n=10; patients with poorly controlled T1D, n=10). Values (median fluorescence intensity) are given as the mean±SEM. Data were analyzed using the two-tailed unpaired Mann-Whitney test (A-C). **p*<0.05, ***p*<0.01 and ****p*<0.001.

CD3⁺CD56⁺ T cell counts in patients with T1D are attributable to activated apoptosis.

Discussion

Despite the evidence that diabetogenic-related T cells promote β -cell death in T1D, it has been challenging to create robust, standardized T cell-based tests (20). One rationale for this is the low frequency of diabetogenic-related T cells in the peripheral circulation of patients with newly diagnosed T1D, as well as the notion that the pathogenic T cells of interest reside in islets or lymph nodes. In this study, we identified ER expansion and inflammasome activation in CD3⁺CD56⁺ T cells as essential biomarkers of patients with T1D. Previous studies demonstrated that CD3⁺CD56⁺ T cells are involved in the pathogenesis of Th1-mediated autoimmune diseases (7) and play a dual role, operating as CD8⁺-specific effector T cells and NK-like cells (21). Furthermore, CD3⁺CD56⁺ T cell counts may be useful for determining disease progression, improving individual stratification for T1D trials and identifying individuals with prediabetes who are at risk during the asymptomatic phase of the disease (13).

Because of their critical involvement in the etiology of T1D, ER stress and the adaptive UPR have recently received substantial attention (16, 17, 22). Calcium levels, protein glycosylation and the redox state in the ER can be altered by excess feeding, viruses, environmental pollutants and chronic inflammation, resulting in aberrant protein folding and

release (23). ER stress is caused by the accumulation of unfolded and misfolded proteins, and cells respond by activating the UPR, an evolutionarily conserved adaptive mechanism (24, 25). When the ER is stressed, the UPR inhibits protein translation and activates signaling pathways that create molecular chaperones to facilitate protein folding or enable misfolded proteins to be destroyed (24, 25). These branches of the UPR activate pro-survival and adaptive signaling in response to acute stress, resulting in the restoration of cellular homeostasis. However, if ER stress is sustained and unresolved, the UPR changes from a pro-adaptive state to a pro-apoptotic factor (26). ER homeostasis disruption can lead to β -cell dysfunction and diabetes mellitus (27). Misfolded insulin has been proven to induce diabetes in both mouse models and humans, supporting this theory (28, 29). Furthermore, mutations in genes critical for ER function induce β -cell failure and early-onset severe diabetes in both experimental animal models and humans (30-33). Similarly, the current study demonstrated that ER dysregulation in the CD3⁺CD56⁺ T cell subset makes them more susceptible to inflammasome activation and apoptosis.

To date, gene-deficient mice have been used to investigate the involvement of the inflammasome-associated protein NLRP3 in modifying susceptibility to T1D (34, 35). NLRP3-deficient NOD mice, as well as wild-type NOD mice treated with an NLRP3 inhibitor, were protected against T1D (35). NLRP3-deficient C57BL/6 mice were likewise protected against diabetes after streptozotocin treatment (34). T cell activation and Th1 cell differentiation were found to be

reduced in NOD mice with NLRP3 deficiency (35). T cells express the chemokines CCR5 and CXCR3, as well as CCL5 and CXCL10 in islets, all of which result in reduced T cell chemotaxis into the islets and protection against T1D pathogenesis (35).

Furthermore, the pancreatic lymph nodes of diabetic NOD animals had higher NLRP3 and pro-IL-1 β expression than prediabetic NOD mice, indicating that inflammasome activation plays a vital role as the disease progresses (34). Although these findings suggest that the inflammasome protein NLRP3 plays a crucial role in the development of T1D, additional research is needed to assess the activity of additional inflammasome-related proteins. To further understand inflammasome participation and regulation during diabetes development, more research is required in both animal models and humans, particularly in individuals with spontaneous autoimmune diabetes. Finally, clarifying the essential actors in inflammasome activation and control will require determining the involvement of inflammasomes in different cell types.

In summary, further research is needed to determine T1D pathogenesis and identify the actual status of CD3⁺CD56⁺ T cells in patients with T1D mediated by high-risk Human Leukocyte antigen (HLA) loci- and non-HLA loci-dependent regulation. These loci indicate pathways that are significant in the development of illness and have been primarily linked to the immune system with the ultimate objective of identifying a probable etiological relationship with T1D.

Conflicts of Interest

All Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

YNH and WDL performed the experiments; YNH, WDL, PHS and DTB analyzed the data; YNH, FJT, CCH and CHW wrote and revised the manuscript. All Authors have approved the manuscript and agreed to be accountable for all aspects of the research to ensure that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

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