Senescent T Cells Predict the Development of Hyperglycemia in Humans

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PATHOPHYSIOLOGY

Senescent T cells have been implicated in chronic inflammatory and cardiovascular diseases. In this study, we explored the relationship between senescent T cells and glycemic status in a cohort of 805 participants by investigating the frequency of $CD57^+$ or $CD28^{null}$ senescent T cells in peripheral blood. Participants with normal glucose tolerance (NGT) with follow-up data (*N* = 149) were included to determine whether hyperglycemia (prediabetes or type 2 diabetes) developed during follow-up (mean 2.3 years). $CD8^+CD57^+$ and $CD8^+CD28^{null}$ T-cell frequencies were significantly higher in prediabetes and type 2 diabetes compared with NGT. Increased $CD57^+$ or $CD28^{null}$ cells in the $CD8^+$ T-cell subset were independently associated with hyperglycemia. Furthermore, among participants with baseline NGT, the frequency of $CD8^+CD57^+$ T cells was an independent predictor of hyperglycemia development. Immunofluorescent analyses confirmed that $CD8^+CD57^+$ T-cell infiltration was increased in visceral adipose tissue of patients with prediabetes or type 2 diabetes compared with those with NGT. Our data suggest that increased frequency of senescent $CD8^+$ T cells in the peripheral blood is associated with development of hyperglycemia.

Immunosenescence is the progressive impairment of the immune system thought to underlie age-related comorbidities (1,2). Change in T-cell immunities is a notable feature of immunosenescence. There is accumulating evidence that senescent T cells are involved in the pathogenesis of cardiovascular diseases (CVDs), including atherosclerosis, acute coronary syndrome, and hypertension (3–7). We reported that hypertensive patients had an increased frequency of senescent $CD8^+$ T cells in peripheral blood, which exhibited CD28 loss and acquisition of CD57 on their surface (8). CD28 loss is a prominent change associated with human aging and is caused by the repetitive antigenic stimulation of T cells. CD57 expression during the late stage of T-cell differentiation might be a distinct measure of senescence

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in T cells (9). Compared with $CD28^+$ or $CD57^{null}$ T cells, $CD28^{null}$ or $CD57^+$ T cells produce more proinflammatory cytokines and exert greater cytotoxicity (10).

Substantial overlap and interconnectivity exists between the etiology and pathophysiology of diabetes and CVD, theorized as the "common soil" hypothesis (11). Despite evidence that immunosenescence can lead to CVD, there are no studies evaluating whether increased T-cell senescence leads to hyperglycemia development in humans. Our study's aim was to investigate the relationship between senescent T cells and glycemic status using a cross-sectional cohort. Furthermore, we studied the longitudinal impact of senescent T cells on hyperglycemia development in participants with normoglycemia.

RESEARCH DESIGN AND METHODS

Study Participants

In this prospective longitudinal study, 805 Koreans registered in the Yonsei Cardiovascular Genome cohort or the Cardiovascular and Metabolic Disease Etiology Research Center-High risk cohort (clinicaltrials.gov: NCT02003781) were recruited via the outpatient clinic of Severance Cardiovascular Hospital from January 2011 to April 2016. Type 2 diabetes was defined as: fasting plasma glucose level ≥126 mg/dL; hemoglobin A_{1c} (HbA_{1c}) ≥6.5% (48 mmol/mol); or history of insulin or oral hypoglycemic agent administration. Participants without diabetes were categorized as either normal glucose tolerance (NGT), defined as fasting plasma glucose levels <100 mg/dL and HbA_{1c} <5.7% (39 mmol/mol) or prediabetes, defined as fasting plasma glucose levels 100–125 mg/dL or HbA_{1c} 5.7–6.4% (39–46 mmol/mol) (12). Hyperglycemia included prediabetes and type 2 diabetes (13). Hypertension was defined as systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg over three visits before the use of antihypertensives. Coronary artery disease was defined as one or more lesions with a >50% diameter reduction by coronary angiography. Patients with any of the following conditions were excluded: significant systemic disease, debilitating malignant disease, severe hypertension (>200/140 mmHg), estimated glomerular filtration rate <30 mL/min/1.73 m^2, and history of overt chronic inflammatory disease and/or receiving anti-inflammatory medications. This study was approved by the Yonsei University College of Medicine Institutional Review Board, and study procedures were in accordance with institutional guidelines (Institutional Review Board number 4-2018-0190). Participants provided informed consent before enrollment.

Blood Glucometabolic Parameters and Incidence of Hyperglycemia

Following an overnight fast, blood tests for biochemical measurements were performed. Serum glucose, insulin, and HbA_{1c} were measured using the Hitachi 7600 analyzer (Hitachi Ltd.), immunoradiometric assay (Insulin-IRMA; DIAsource, Louvain-la-Neuve, Belgium), and immunoassay by an Integra 800 CTS (Roche Diagnostics), respectively. We examined medical records of participants with normoglycemia who had at least one clinic visit for any reason from the date of registry enrollment until December 2016. Of 222 NGT participants, follow-up blood glucose data were available in 149 (88 men and 61 women). Based on fasting plasma glucose or HbA_{1c} levels, participants with baseline NGT were defined as hyperglycemia progressors when those met diagnostic criteria for prediabetes or diabetes in at least one time interval.

Immunophenotype Analysis of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and immediately stained for flow cytometry analyses. PBMCs were incubated with directly conjugated monoclonal antibodies for 20 min at 4°C using anti-CD3 (Horizon V500), anti-CD4 (phycoerythrin [PE]-Cy7), anti-CD8 (allophycocyanin [APC]-H7), anti-CD19 (PerCP-Cy5.5), anti-CD28 (APC) (all from BD Biosciences, San Jose, CA), and anti-CD57 (eFluor 450; BioLegend, San Diego, CA). Multicolor flow cytometry was performed using an LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star, San Carlos, CA). The gating strategy is provided in Supplementary Fig. 1. FlowJo software autogating was performed on CD28 and CD57 T cells. Frequency of $CD8^+CD57^+$ or $CD8^+CD28^{null}$ T cells and $CD4^+CD57^+$ or $CD4^+CD28^{null}$ T cells was expressed as a percentage of the entire population of $CD8^+$ and $CD4^+$ T cells.

In Vitro Stimulation of T Cells and Intracellular Cytokine Staining

Cytomegalovirus (CMV) serostatus was evaluated using a chemiluminescent microparticle immunoassay (Abbott Laboratories, Chicago, IL), and a titer of ≥6.0 antibody units/mL of IgG was considered CMV IgG-seropositive. PBMCs were stimulated with overlapping peptides from CMV pp65 (0.6 nmol of each peptide/mL) (Miltenyi Biotec) for 6 h in the presence of PE-conjugated anti-CD107a (BD Biosciences). After 1 h of incubation, brefeldin A (GolgiPlug; BD Biosciences) and monensin (GolgiStop; BD Biosciences) were added to accumulate cytokine proteins intracellularly. Following surface staining with anti-CD3 (Horizon V500), anti-CD4 (PerCP-Cy5.5), anti-CD8 (APC-H7), anti-CD28 (Horizon V450), and anti-CD57 (APC), the cells were fixed and permeabilized using a Fixation/Permeabilization Buffer Kit and further stained for intracellular cytokines with anti–interferon-γ (FITC) and anti–tumor necrosis factor-α (PE-Cy7) (both from BD Biosciences). All samples were assessed using an LSR II Flow Cytometer (BD Biosciences), and the data were analyzed using FlowJo software.

Adipose Tissue Immunohistochemistry and Immunofluorescence Staining

Omental adipose tissue from consenting patients without acute infection or receiving immune-modulating medications

was obtained during abdominal surgery (e.g., hepatectomy, colectomy, or cholecystectomy due to cancer). In order to identify infiltrated $CD8^+$ T cells adjacent to macrophages within adipose tissues, immunohistochemistry was performed with formalin-fixed, paraffin-embedded sections using the primary antibodies of CD68 (Thermo Fisher Scientific, Waltham, MA) and CD8 (Abcam, Cambridge, U.K.) and the secondary antibody of Polink DS (GBI Labs, Bothell, WA). Next, double immunofluorescence staining was performed to colocalize $CD57^+$ with $CD8^+$ T cells. Primary antibodies used were CD57 (HNK-1, Leu-7, and MA5-11605; Thermo Fisher Scientific) and CD8 (ab4055; Abcam). Secondary antibodies used were Alexa Fluor 488 (A21042; Life Technologies, Carlsbad, CA) and Alexa Fluor 647 (A21245; Life Technologies). Slides were examined on a Zeiss AXIO Imager A1 & HBO100 (Zeiss, Oberkochen, Germany) using AxioVision software (Zeiss).

Statistical Analyses

All statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corp., Armonk, NY). All *P* values of <0.05 were considered significant.

RESULTS

Participants' baseline characteristics are summarized in Table 1. According to the glycemic status, age, BMI, and HOMA of insulin resistance (HOMA-IR) were increased, whereas HOMA of pancreatic β-cell function (HOMA-β) was decreased. $CD8^+CD57^+$ and $CD8^+CD28^{null}$ T cells were significantly increased in patients with prediabetes and type 2 diabetes compared with those with NGT (Fig. 1*A* and *B*). However, senescent $CD4^+CD57^+$ or $CD4^+CD28^{null}$ T-cell frequency was not significantly different (Fig. 1*C* and *D*). In Supplementary Fig. 2, representative flow cytometry plots present CD57 and CD28 expression in the $CD8^+$

Table 1—Baseline characteristics of the study participants according to glycemic status (*N* = 805)

Baseline characteristics	NGT (*N* = 222)	Prediabetes (*N* = 302)	Type 2 diabetes (*N* = 281)	*P* value
Demographics				
Age (years)	56.5 (48.0–67.0)	62.0 (54.0–69.0)	64.0 (58.0–70.0)	<0.001
Male sex [*n* (%)]	125 (56.3)	215 (71.2)	184 (65.5)	0.002
BMI (kg/m^2)	24.5 (22.5–26.7)	25.5 (23.2–27.6)	25.6 (24.0–27.9)	<0.001
Waist circumference (cm)	85.0 (79.0–91.3)	87.9 (82.0–93.3)	89.0 (83.0–94.0)	<0.001
Hypertension [*n* (%)]	130 (58.6)	257 (85.1)	242 (86.1)	<0.001
Systolic blood pressure (mmHg)	126.3 (114.8–136.5)	129.2 (120.0–139.0)	129.5 (119.0–141.3)	0.014
Diastolic blood pressure (mmHg)	79.0 ± 10.4	79.3 ± 10.6	77.3 ± 10.3	0.046
Hyperlipidemia [*n* (%)]	94 (42.3)	173 (57.3)	154 (54.8)	0.002
Coronary artery disease [*n* (%)]	51 (23.0)	141 (46.7)	151 (53.7)	<0.001
Glucose-lowering drug use* [*n* (%)]	—	—	104 (37.0)	—
Laboratory indices				
Fasting glucose (mg/dL)	92.0 (85.0–98.0)	99.0 (92.0–107.0)	117.0 (100.0–134.0)	<0.001
HbA_{1c} (%)	5.50 (5.40–5.70)	5.80 (5.60–6.00)	6.80 (6.30–7.30)	<0.001
HbA_{1c} (mmol/mol)	36.6 (35.5–38.8)	39.9 (37.7–42.1)	50.8 (45.4–56.3)	<0.001
AST (IU/L)	21.0 (18.0–27.0)	23.0 (19.0–28.0)	23.0 (18.0–27.0)	0.030
ALT (IU/L)	16.0 (12.0–24.5)	20.0 (14.0–27.0)	19.0 (14.0–28.0)	<0.001
BUN (mg/dL)	14.9 (12.6–17.7)	16.2 (13.4–19.9)	16.9 (13.6–20.2)	<0.001
Creatinine (mg/dL)	0.89 (0.77–1.05)	0.92 (0.77–1.11)	0.97 (0.80–1.14)	0.053
eGFR MDRD (mL/min/1.73 m^2)	82.8 (70.8–96.3)	84.1 (66.9–98.9)	79.8 (65.1–92.9)	0.034
Total cholesterol (mg/dL)	175.0 (153.8–203.0)	164.0 (141.0–190.5)	155.5 (133.0–178.8)	<0.001
Triglycerides (mg/dL)	101.5 (73.0–153.5)	115.0 (85.0–163.0)	119.0 (87.0–174.0)	0.002
HDL cholesterol (mg/dL)	51.0 (43.0–59.0)	46.0 (41.0–56.0)	46.0 (39.0–52.0)	<0.001
LDL cholesterol (mg/dL)	102.0 (79.0–124.0)	87.1 (69.1–110.0)	81.0 (64.1–100.8)	<0.001
Uric acid (mg/dL)	5.10 (4.10–6.15)	5.50 (4.50–6.60)	5.20 (4.30–6.30)	0.041
WBC count (10^3/μL)	5.95 (4.85–7.23)	6.19 (5.13–7.30)	6.42 (5.55–7.39)	0.012
HOMA-β (%)	141.0 ± 199.6	124.6 ± 91.6	85.6 ± 66.5	<0.001
HOMA-IR	2.19 ± 1.11	2.85 ± 1.97	3.38 ± 2.29	<0.001
Frequency of T-cell subset				
$CD57^+$ cells in $CD4^+$ T cells (%)	4.92 (3.10–7.37)	4.80 (3.40–7.23)	5.00 (3.10–7.83)	0.896
$CD28^{null}$ cells in $CD4^+$ T cells (%)	3.00 (1.20–7.15)	3.50 (1.30–9.33)	3.66 (1.50–9.55)	0.227
$CD57^+$ cells in $CD8^+$ T cells (%)	38.7 ± 16.6	46.6 ± 16.1	47.8 ± 16.9	<0.001
$CD28^{null}$ cells in $CD8^+$ T cells (%)	39.5 ± 19.5	48.2 ± 18.3	49.0 ± 18.2	<0.001

Continuous variables are described as mean ± SD for parametric variables and median (interquartile range) for nonparametric variables unless otherwise indicated. The *P* values were calculated using the Kruskal-Wallis test or one-way ANOVA for continuous variables and χ^2 tests for categorical variables. BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; WBC, white blood cell. *Oral hypoglycemic agents or insulin. HOMA-β = (fasting serum insulin [μU/mL] × 20)/(fasting serum glucose [mmol/L] − 3.5); HOMA-IR = (fasting serum insulin [μU/mL] × fasting serum glucose [mmol/L])/22.5.

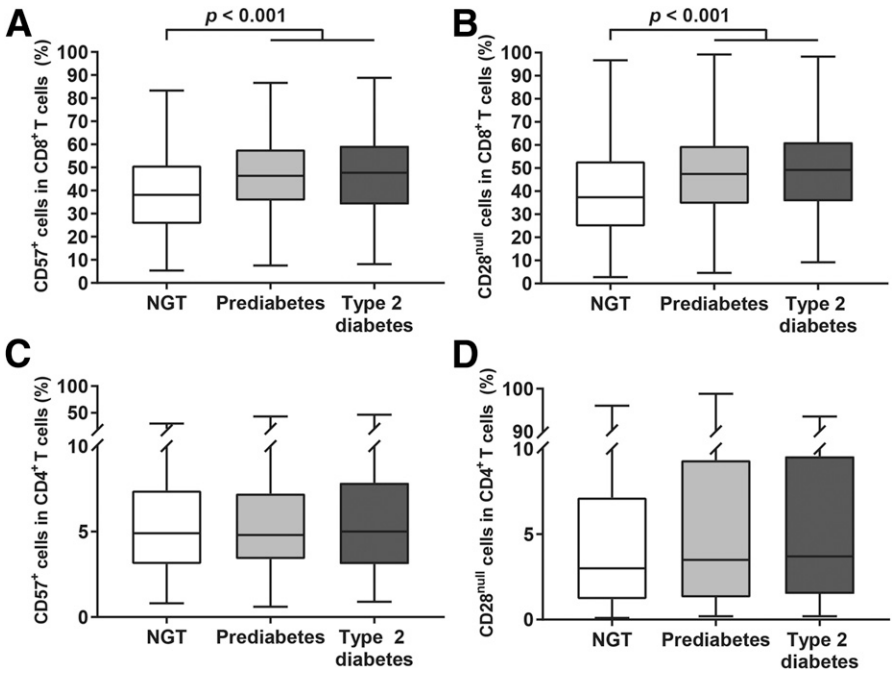


Figure 1—The relative frequency of senescent T cells in the peripheral blood from subjects with NGT (*N* = 222), prediabetes (*N* = 302), and type 2 diabetes (*N* = 281). Both CD57$^+$ and CD28^{null} fractions are significantly increased in CD8$^+$ T cells of participants with prediabetes and type 2 diabetes (*A* and *B*) but not in CD4$^+$ T cells (*C* and *D*). The horizontal line in the middle of each box indicates the median, the top and bottom borders of the box mark the 75th and 25th percentiles, respectively, and the top and bottom whiskers mark the maximum and minimum values, respectively.

T-cell subset in the groups. In simple correlation analyses, fasting plasma glucose levels correlated significantly with frequency of CD8$^+$CD57$^+$ and CD8$^+$CD28^{null} T cells (Supplementary Table 1). There was a positive correlation between CD8$^+$CD57$^+$ T-cell frequency and HOMA-IR. Logistic regression analyses were conducted to determine odds ratios for predicting hyperglycemia (Table 2). After adjustment for traditional diabetes risk factors, frequencies of both CD8$^+$CD57$^+$ and CD8$^+$CD28^{null} T cells were significantly associated with hyperglycemia in the cross-sectional data set (*N* = 805). When divided into two groups based upon tertiles of CD8$^+$CD57$^+$ T-cell frequency

Table 2—Multiple logistic regression models for independent determinants of the presence of hyperglycemia (*N* = 805)

	Univariate			Multivariate		
Variables	OR	95% CI	*P* value	OR	95% CI	*P* value
Model 1 for CD8$^+$CD57$^+$ T cells						
CD8$^+$CD57$^+$ T cells (%)	1.697	1.436–2.006	<0.001	1.415	1.160–1.727	0.001
Age, per year	1.052	1.036–1.067	<0.001	1.018	1.000–1.037	0.052
Sex (0 = male, 1 = female)	0.594	0.433–0.816	0.001	0.723	0.493–1.059	0.096
BMI (kg/m^2)	1.114	1.059–1.172	<0.001	1.074	1.015–1.136	0.013
Hypertension (0 = no, 1 = yes)	4.204	2.953–5.985	<0.001	3.686	2.409–5.641	<0.001
Hyperlipidemia (0 = no, 1 = yes)	1.739	1.273–2.377	0.001	1.399	0.974–2.010	0.069
Coronary artery disease (0 = no, 1 = yes)	3.364	2.365–4.786	<0.001	3.838	2.542–5.796	<0.001
WBC count (10^3/μL)	1.135	1.030–1.251	0.010	1.090	0.980–1.213	0.114
Model 2 for CD8$^+$CD28^{null} T cells						
CD8$^+$CD28^{null} T cells (%)	1.671	1.411–1.979	<0.001	1.384	1.145–1.674	0.001
Age, per year	1.052	1.036–1.067	<0.001	1.022	1.004–1.040	0.017
Sex (0 = male, 1 = female)	0.594	0.433–0.816	0.001	0.648	0.446–0.942	0.023
BMI (kg/m^2)	1.114	1.059–1.172	<0.001	1.073	1.014–1.135	0.014
Hypertension (0 = no, 1 = yes)	4.204	2.953–5.985	<0.001	3.557	2.319–5.456	<0.001
Hyperlipidemia (0 = no, 1 = yes)	1.739	1.273–2.377	0.001	1.349	0.940–1.936	0.105
Coronary artery disease (0 = no, 1 = yes)	3.364	2.365–4.786	<0.001	3.827	2.539–5.768	<0.001
WBC count (10^3/μL)	1.135	1.030–1.251	0.010	1.081	0.972–1.203	0.152

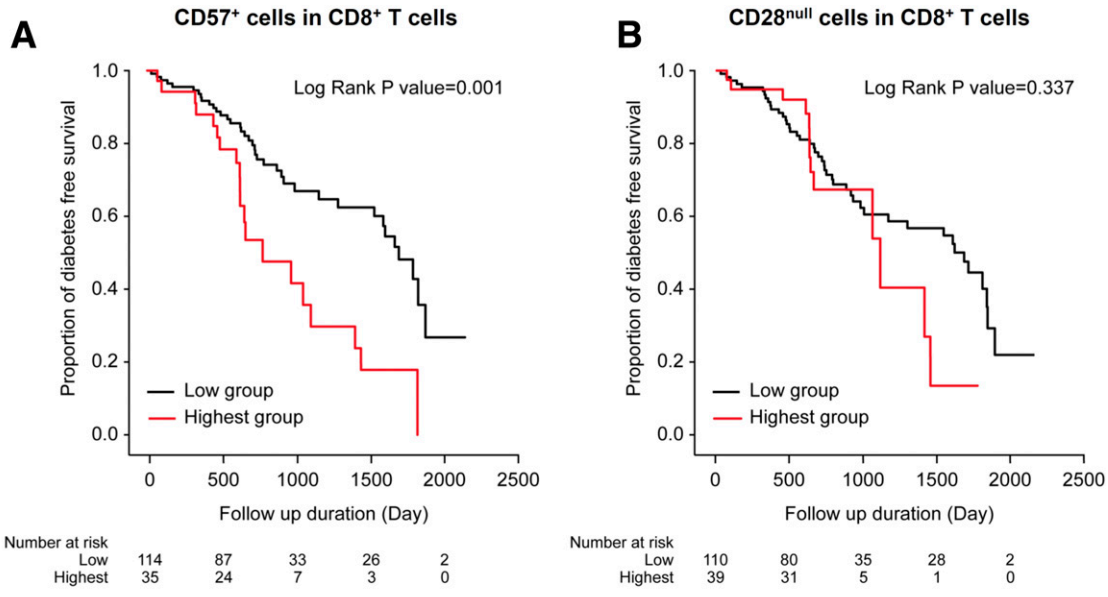
OR, odds ratio; WBC, white blood cell.

in peripheral blood, HOMA-IR but not HOMA-β was significantly different in the higher (second to third) tertiles compared with the lower (first) tertile (Supplementary Table 2). When a cutoff of ≥2.34 for HOMA-IR was chosen (14), $CD8^+CD57^+$ T-cell frequencies were independently associated with insulin resistance (Supplementary Table 3).

Of 149 NGT participants, 58 developed prediabetes or type 2 diabetes during follow-up (2.32 ± 1.45 years). NGT participants were divided into two groups based upon tertiles of $CD8^+CD57^+$ or $CD8^+CD28^{null}$ T-cell frequency: low (first to second tertile) and high (third tertile). In the high $CD8^+CD57^+$ T-cell group, 57% (20 out of 35) developed hyperglycemia compared with 33% (38 out of 114) in the low group. We observed a significant difference in incidence rates among participants with a high versus low frequency of $CD8^+CD57^+$ T cells (Kaplan-Meier and log-rank test, P = 0.001) (Fig. 2*A*). However, this difference was not found for $CD8^+CD28^{null}$ T cells (Fig. 2*B*). A Cox regression model (Fig. 2*C*) revealed that, after adjusting for traditional risk factors for diabetes, the hazard ratio for developing hyperglycemia per percentage of $CD8^+CD57^+$ T cells was 1.785 (95% CI 1.298–2.455).

In humans, CMV is known as an important antigen for repetitive T-cell stimulation and is involved in the accumulation of $CD28^{null}$ or $CD57^+$ senescent T cells (9). Therefore, we analyzed CMV-specific antigen reactivity of T cells. Proinflammatory and cytotoxic functions of T cells were evaluated and compared among 56 baseline NGT participants who did versus did not develop hyperglycemia (Supplementary Table 4). All participants were seropositive for CMV in the current study (data not shown). CMV pp65-specific CD107a-expressing $CD8^+$ T cells, which represent degranulation of cytotoxic proteins such as perforin and granzymes (15), were more frequently observed in those who did compared with those who did not progress to hyperglycemia.

Adipose tissue is a major target of insulin action and immune cell reservoir (16). Using immunohistochemistry, we identified infiltrated $CD8^+$ T cells adjacent to macrophages within adipose tissues (Supplementary Fig. 3*A*). Furthermore, immunofluorescent analyses showed histological evidence of greater $CD8^+CD57^+$ T-cell infiltration in omental adipose tissues of patients with prediabetes or type 2 diabetes compared with those with NGT (Supplementary Fig. 3*B* and *C*).



C

	Hazard ratios (95% CI)
	Percent $CD8^+CD57^+$ T cells (%)
Model 1	1.728 (1.313-2.276)
Model 2	1.655 (1.241-2.207)
Model 3	1.785 (1.298-2.455)

Figure 2—Increased frequency of $CD8^+CD57^+$ T cells predicts development of hyperglycemia in participants with NGT (N = 149). Kaplan-Meier curves for hyperglycemia development according to the highest tertile vs. the lower two tertiles of the frequency of $CD8^+CD57^+$ (*A*) or $CD8^+CD28^{null}$ (*B*) T cells. *C*: Cox regression analyses for incidence of hyperglycemia. Model 1, unadjusted; model 2, adjusted for age and sex; model 3, adjusted for age, sex, BMI, hypertension, hyperlipidemia, coronary artery disease, and white blood cell count.

DISCUSSION

The current study demonstrates that increased senescent $CD8^+$ T cells in peripheral blood are independently associated with prevalence and incidence of prediabetes or type 2 diabetes. Furthermore, increased frequency of $CD8^+CD57^+$ T cells was associated with insulin resistance, and we observed histological evidence of $CD8^+CD57^+$ T-cell infiltration in visceral adipose tissues (VATs) of patients with prediabetes or type 2 diabetes.

Although type 2 diabetes pathogenesis is not fully understood, insulin resistance is the hallmark (17). Obesity is associated with chronic low-grade inflammation in VATs and a sustained whole-body proinflammatory state (18). Infiltration of T cells into adipose tissue has been extensively reported (19,20). Nishimura et al. (21) found large numbers of $CD8^+$ effector T cells in epididymal adipose tissue in mice fed a high-fat diet. Immunologic/genetic depletion of $CD8^+$ T cells lowered macrophage infiltration and adipose tissue inflammation and ameliorated systemic insulin resistance. In a human study that included patients with diabetes, $CD4^+$ lymphocytes in VAT biopsies correlated significantly with BMI (19). In the current study, senescent $CD8^+$ T cells and not $CD4^+$ T cells were significantly associated with present and future hyperglycemia.

Common persistent viral infections (especially human CMV) are attributed to increasing $CD8^+CD28^{null}$ ($CD8^+CD57^+$) T-cell populations with age (9) and have been shown to express perforin, granzymes, and granulysin, with high cytotoxic potential (8,22,23). This finding aligns with our previous observation that CMV pp65-specific interferon-γ–, tumor necrosis factor-α–, and CD107a-expressing cells were more frequently observed in $CD8^+CD57^+$ T cells compared with $CD8^+CD57^-$ T cells (24). Previous studies demonstrated that proinflammatory cytokines could promote insulin resistance (25). Correspondingly, we found that increased frequency of $CD8^+CD57^+$ T cells was independently associated with insulin resistance measured by HOMA-IR. In addition, among participants with baseline NGT, CMV pp65-specific CD107a-expressing $CD8^+$ T cells were more frequently observed in those who did versus did not progress to hyperglycemia. How senescent T cells interact with other immune cells within target tissues (including adipose) in hyperglycemia development remains to be investigated. Although our findings suggest that senescent T cells might be involved in the early development of diabetes, we were unable to elucidate whether senescent T cells are indicators or pathogenic players for hyperglycemia.

In conclusion, present and future hyperglycemia (prediabetes or type 2 diabetes) were associated with increased senescent $CD8^+$ T cells in peripheral blood. Larger prospective studies and sophisticated mechanistic experiments are warranted to confirm whether modulation of immunosenescence is a potential new therapeutic target of diabetes.

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