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PRECLINICAL RESEARCH

Circulating Pro-Vascular Progenitor Cell Depletion During Type 2 Diabetes

Translational Insights Into the Prevention of Ischemic Complications in Diabetes

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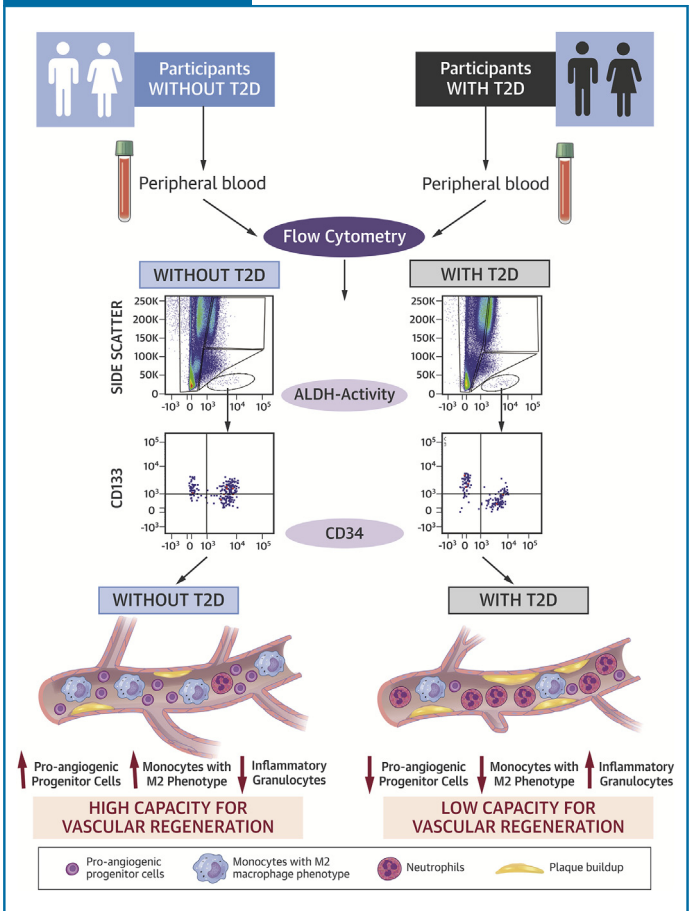
VISUAL ABSTRACT



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HIGHLIGHTS

- This study combined ALDH activity with cell surface marker expression to develop a multiparametric flow cytometry assay to assess proangiogenic progenitor and proinflammatory cell content in the peripheral blood of patients with T2D compared with age-matched control subjects.
- Patients with T2D exhibited an increased frequency of proinflammatory ALDH^{hi} cells with granulocyte side scatter properties and a decreased frequency of circulating monocytes with an M2 phenotype that is associated with proangiogenic and anti-inflammatory functions.
- Patients with T2D exhibited significant depletion of circulating provascular ALDH^{hi}CD34+ progenitor cells with primitive, migratory, endothelial, and pericyte phenotypes.
- Subgroup analyses that stratified patients with T2D according to age, duration of T2D, insulin requirement, and glycosylated hemoglobin levels revealed that only the duration of T2D correlated with vascular progenitor cell depletion.
- Flow cytometric assessment of circulating ALDH^{hi} cell subsets represents a promising translational approach for identifying patients with T2D at increased risk for cardiovascular comorbidities.

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SUMMARY

Detection of vascular regenerative cell exhaustion is required to combat ischemic complications during type 2 diabetes mellitus (T2D). We used high aldehyde dehydrogenase (ALDH) activity and surface marker co-expression to develop a high-throughput flow cytometry–based assay to quantify circulating proangiogenic and proinflammatory cell content in the peripheral blood of individuals with T2D. Circulating proangiogenic monocytes expressing anti-inflammatory M2 markers were decreased in patients with T2D. Individuals with longer duration of T2D exhibited reduced frequencies of circulating proangiogenic ALDH^{hi}CD34+ progenitor cells with primitive (CD133) and migratory (CXCR4) phenotypes. This approach consistently detected increased inflammatory cell burden and decreased provascular progenitor content in individuals with T2D.

ABBREVIATIONS AND ACRONYMS

ALDH = aldehyde dehydrogenase

BM = bone marrow

HbA$_{1c}$ = glycosylated hemoglobin

ROS = reactive oxygen species

SSC = side scatter

T2D = type 2 diabetes mellitus

Wnt = wingless related integration site

Approximately 400 million individuals worldwide experience type 2 diabetes (T2D), and this number is expected to rise to >600 million by 2045 (1-3). Although various mechanisms have been suggested to mediate the vascular complications of diabetes, there is growing interest in the theory that diabetes may lead to chronic inflammation, which in turn increases oxidative stress on vascular regenerative cells, inciting a state of vasculopenia. This damaging microenvironment also contributes to the death and dysfunction of bone marrow (BM)-derived and circulating proangiogenic progenitor cells, leading to an inability to respond to vessel damage (4). Thus, ongoing endothelial damage combined with reduced blood vessel regenerative capacity in patients with T2D culminates in a 2- to 5-fold increased risk for the development of ischemic cardiovascular diseases, including critical limb ischemia, myocardial infarction, and stroke (1,3). Although newer antihyperglycemic agents reportedly improve cardiovascular outcomes in diabetes (5–15), the unmet need and residual risk remain prohibitively high in T2D (16).

To minimize the risks associated with reduced blood flow causing ischemia, multiple endogenous mechanisms can be activated to reverse vascular dysfunction (4). These multicellular processes include vasculogenesis, the creation of de novo vessels from endothelial progenitor cells; angiogenesis, the sprouting of new blood vessels from pre-existing vessels; and arteriogenesis, the beneficial remodeling of pre-existing collateral vessels to form a "natural bypass" toward the ischemic region (4,17). Although angiogenesis and postnatal vasculogenesis have been widely studied, both processes can be limited in adults by the scarcity of circulating provascular progenitor cells of hematopoietic and endothelial lineages (18,19). Although arteriogenesis is not as well understood, accessory immune cells (including monocytes and macrophages) are recruited to pre-existing collateral vessels and participate in vessel remodeling to activate blood flow (4,18,20,21). Thus, these processes rely on structural and secretory contributions from circulating hematopoietic and endothelial cells that originate from the BM (22,23). In the context of T2D, the impact of glucotoxicity and

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All authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* author instructions page.

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increased oxidative stress on the frequency and function of these regenerative progenitor cells is not well understood.

Aldehyde dehydrogenase (ALDH) is an intracellular detoxification enzyme highly expressed in progenitor cells with documented proangiogenic secretory function (17). ALDH acts to protect long-lived cells from oxidative stress by metabolizing toxic alkylating aldehyde agents, which can lead to cellular damage. In addition, ALDH is the rate-limiting enzyme in the intracellular production of retinoic acid, a potent morphogen. Thus, as progenitor cells differentiate toward a mature phenotype, ALDH-activity is reduced. Our group and others have previously documented the proangiogenic signaling capacity of ALDH^{hi} progenitor cells from BM and umbilical cord blood (17,24,25).

BM cells of patients with T2D exhibit reduced expression of markers associated with proangiogenic progenitor cells (CD34 and CD133) due to premature differentiation accelerated by hyperglycemia and increased oxidative stress (18,23,26). The T2D BM microenvironment also exhibits increased cell turnover, lending to heightened inflammatory responses and inhibited distribution of provascular progenitor cells to ischemic tissues (23,27). The amplified inflammation leads to increased NADPH oxidase-1 function, which significantly elevates intracellular reactive oxygen species (ROS) formation (28). The examination of circulating progenitor cell content in the peripheral circulation may confirm the extent of this process (termed "regenerative cell exhaustion") and illuminate the therapeutic implications of BM dysfunction on vascular regeneration.

The goal of this study was to assess the balance between circulating vascular regenerative progenitor cells and inflammatory cells in patients with T2D. We used the detection of high ALDH-activity according to flow cytometry to quantify the prevalence of circulating progenitor cells in the peripheral blood of patients with T2D and age-matched control subjects. High ALDH-activity in conjunction with 6-color cell surface marker analyses allowed us to quantify the frequencies of proangiogenic and inflammatory cell types that affect the repair of ischemic injury in patients with T2D. Patients with T2D exhibited a significant decrease in circulating cells with hematopoietic and endothelial progenitor cell phenotype. In addition, circulating monocytes with an anti-inflammatory M2 phenotype were decreased in patients with T2D, and primitive granulocytes with proinflammatory function were significantly increased in patients with T2D, suggesting a shift toward a proinflammatory phenotype (29). These studies provide a foundation to assess vascular regenerative cell content during the progression of T2D and may be developed as a surrogate assay to estimate the capacity to mitigate ischemia via a provascular regenerative response.

METHODS

PATIENT CHARACTERISTICS. A total of 30 individuals >40 years of age with established T2D of >5 years were age- and sex-matched with 30 individuals without T2D. Written informed consent was provided, and all studies were approved prior to study initiation by the Advarra central institutional review board.

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS. Up to 50 ml of peripheral blood was drawn from each patient into ethylenediaminetetraacetic acid-lined blood collection tubes. Cells were layered on Hypaque-Ficoll solution placed in SepMate tubes (STEMCELL Technologies, Vancouver, British Columbia, Canada) to aid in the removal of red blood cells. Any red blood cells remaining were lysed with ammonium chloride and washed in phosphate-buffered saline to remove cellular debris.

ANALYSES OF PROGENITOR CELLS. Peripheral blood mononuclear cells were examined for ALDH-activity by using Aldefluor reagent (STEMCELL Technologies) following the manufacturer's instructions (17,24,25). Briefly, cells were incubated at 37°C for 30 min with Aldefluor reagent. Subsequently, cells were centrifuged, washed with phosphate-buffered saline, and resuspended in ALDH buffer to block the efflux of the fluorescent substrate via adenosine triphosphate-binding cassette transporters. Next, cells were labeled with fluorochrome-conjugated, anti-human antibodies to surface markers marking primitive (CD34 and CD133) and more mature (CD33 and CD45) hematopoietic cells, endothelial cells (CD31, CD146, and CD144), monocytes (CD14), M1/M2 phenotype (CD68, CD80, and CD163), and granulocyte (CD15, CD16b, and CD66b) phenotypes. Antibodies were from Becton Dickinson, Miltenyi, and BioLegend, as specified in Supplemental Table 1. Cells were incubated with the antibodies for 30 min at 2°C to 8°C and washed in phosphate-buffered saline to ensure that excess antibodies were removed from the sample. Circulating progenitor cell content was assessed via 6-color, multiparametric flow cytometry on a BD LSRFortessa X-20 cytometer (BD Biosciences (Franklin Lakes, New Jersey) and analyzed with the FlowJo version 10 software (FlowJo, LLC, Ashland, Oregon). Side scatter (SSC) property, a measure of light scatter due to

intracellular complexity or granularity, was used to further identify cells with low, intermediate, or high intracellular complexity. A minimum of 10^6 events was collected for every sample, assuring the analysis of >500 cells in the rare ALDH^{hi}SSC^{low} population.

STATISTICAL ANALYSIS. Statistical analyses were performed with the Student's *t*-test for comparison of results from the group with T2D versus the age-matched control individuals and for analyses of patients with T2D (n = 30) stratified into subgroups for assessment of correlations with sex (male, n = 12; female, n = 18), HbA_{1c} values (HbA_{1c} ≤7, HbA_{1c} >7, n = 15), insulin use (no insulin, n = 18; on insulin, n = 12), age (≤70 years, n = 16; >70 years, n = 14), and duration of T2D (≤13 years, n = 15; >13 years, n = 15) on relevant circulating cellular subpopulations. Data from all 30 patients with T2D were included in the subgroup analyses. The use of nonparametric tests or permutation tests was not required.

TABLE 1 Baseline Characteristics of the Study Population

	Control Subjects (n = 30)	Patients With T2D (n = 30)
Age, yrs	72.3 ± 1.6	71.4 ± 1.7
Male/female	12 (40)/18 (60)	12 (40)/18 (60)
Duration of T2D, yrs	NA	14.0 ± 0.9
HbA_{1c}, %	5.5 ± 0.1	7.2 ± 0.2*
LDL-C, mmol/L	2.5 ± 0.2	1.5 ± 0.1*
HDL-C, mmol/L	1.4 ± 0.1	1.3 ± 0.1
Total cholesterol, mmol/L	4.5 ± 0.2	3.5 ± 0.2*
Hypertensive therapy	24 (80)	26 (87)
Statin therapy	15 (50)	22 (73)
Metformin	0 (0)	23 (77)
Insulin	0 (0)	12 (40)

Values are mean ± SEM or n (%). *p < 0.001 with the Student's *t*-test.
HbA_{1c} = glycosylated hemoglobin; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; NA = not applicable; T2D = type 2 diabetes mellitus.

RESULTS

DEMOGRAPHIC AND BIOCHEMICAL CHARACTERISTICS. Baseline patient characteristics as well as their clinical histories are shown in **Table 1**. As expected, HbA_{1c} levels were higher in patients with T2D. The average duration of diabetes in this cohort was 14.0 ± 0.9 years, and the average age was 71.4 ± 1.7 years. The age and percent ratio of male to female patients (40:60) were balanced between the T2D cohort and matched control subjects. The frequency of patients taking an antihypertensive agent was equivalent in both populations. High-density lipoprotein cholesterol levels were similar between the groups, although total cholesterol and low-density lipoprotein cholesterol levels were significantly lower in patients with T2D. The lower total cholesterol and low-density lipoprotein cholesterol levels are likely due to greater use of statins within the T2D cohort (22 of 30) compared with the control group (15 of 30). The majority of the patients with T2D (77%) were taking metformin, and insulin was used by 12 (40%) of 30 patients with T2D.

CIRCULATING CELLS WITH PROANGIOGENIC MONOCYTE AND ANTI-INFLAMMATORY M2 PHENOTYPES WERE DECREASED IN PATIENTS WITH T2D. We first examined the relative expression of cell surface markers associated with hematopoietic, endothelial, monocyte, and granulocyte phenotypes. Importantly, previous studies have shown the functional relevance of these cell types in the coordination of proangiogenic responses after transplantation (30–33). When gated on total cellular events and analyzed for single-cell surface marker expression, the frequency of circulating cells expressing primitive hematopoietic and endothelial cell-associated surface markers was equivalent in patients with T2D and the matched control subjects (**Table 2**, Supplemental Figures 1A to 1H). In contrast, the frequencies of circulating cells expressing the monocyte marker CD14 or the M2 polarization marker CD163 were significantly decreased in patients with T2D (**Table 2**, Supplemental Figures 1I to 1L); the frequency of cells expressing CD80, a marker associated with the M1 phenotype, was increased in patients with T2D. Collectively, the diminished frequency of provascular CD14+ circulating monocytes combined with a shift from the M2 to M1 phenotype suggested increased inflammation in patients with T2D. In addition, more sensitive analyses using multiple markers combined with ALDH-activity was required to accurately detect differences in circulating proangiogenic progenitor cell frequencies.

ALDH^{hi}SSC^{hi} GRANULOCYTIC CELLS WERE INCREASED IN PATIENTS WITH T2D. To more definitively assess circulating proangiogenic progenitor cell frequencies in the peripheral blood of patients with T2D, the Aldefluor assay was used to detect circulating cells with high ALDH-activity, a conserved characteristic in multiple progenitor cell lineages. We have previously documented the robust proangiogenic secretory function of ALDH^{hi} cells from human umbilical cord blood and BM (17,24,25,34). We identified three distinct cell populations with high ALDH-activity segregated further according to SSC properties representing cells with increasing intracellular complexity. A reversible inhibitor of ALDH-activity, *N,N*-diethylaminobenzaldehyde, was used to discern cells with low versus high ALDH-activity (**Figure 1A**), alongside low (R1) versus intermediate (R2) versus

TABLE 2 Circulating Monocytes With Anti-inflammatory M2 Phenotypes Were Decreased in Patients With T2D

	Marker	Control	T2D	p Value	Description/Expression
Hematopoietic	CD45	91.1 ± 2.2	90.4 ± 2.2	0.82	Pan-leukocyte marker or leukocyte common antigen • Expressed on all hematopoietic cells except erythrocytes
	CXCR4/CD184	83.8 ± 1.3	82.4 ± 2.4	0.61	CXC chemokine receptor type 4 or fusin • Expressed on hematopoietic cells with migratory function
	CD33	49.4 ± 2.5	49.2 ± 2.4	0.95	Sialic acid binding IgG-like lectin 3 of Siglec-3 • Expressed on primitive cells of the myeloid lineage
	CD34	3.7 ± 0.7	2.9 ± 0.6	0.38	Sialomucin, adhesion to matrix and stromal cells in the bone marrow • Expressed on hematopoietic/endothelial progenitor cells
Endothelial	CD31	73.0 ± 1.9	72.3 ± 1.9	0.74	Platelet endothelial cell adhesion molecule (PECAM-1) • Expressed on monocytes, neutrophils, and endothelial cells
	CD144	44.0 ± 2.8	43.6 ± 2.5	0.91	Cadherin 5, type 2, or vascular endothelial–cadherin • Expressed on endothelial cells and some granulocytes
	CD146	1.4 ± 0.3	1.1 ± 0.2	0.40	Melanoma cell adhesion molecule (MCAM) or mucin 18 • Expressed on endothelial cells and pericytes
	CD133	1.5 ± 0.4	1.3 ± 0.3	0.57	Prominin-1, pentaspan transmembrane protein • Expressed on hematopoietic/endothelial progenitor cells
Monocyte	CD14	10.1 ± 1.7	5.9 ± 0.8	0.05	Co-receptor for bacterial lipopolysaccharide • Expressed on monocytes, macrophages, some granulocytes
	CD68	42.2 ± 3.0	43.6 ± 2.6	0.73	Macrosialin, scavenger receptor class D, member 1 • Expressed on monocytes and macrophages
M1 phenotype	CD80	3.6 ± 0.6	5.2 ± 0.5	0.04	B7-1, ligand for CD28 and CTLA-4 • Expressed on pro-inflammatory M1 macrophages
M2 phenotype	CD163	14.5 ± 1.0	10.8 ± 0.7	0.003	Low-affinity scavenger receptor for hemoglobin-haptoglobin • Expressed on anti-inflammatory M2 macrophages

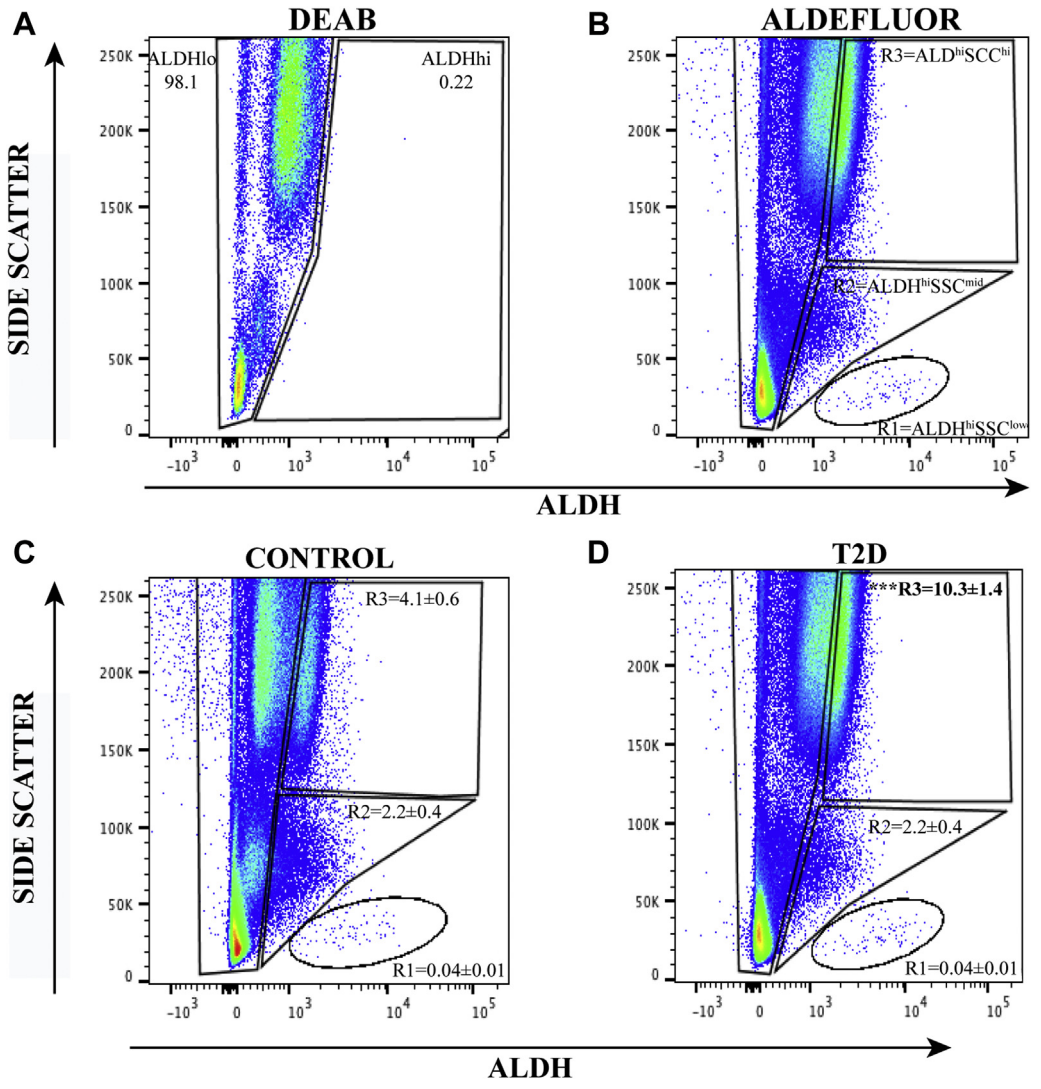
The frequency of cells expressing mature and primitive hematopoietic and endothelial markers was equal in patients with type 2 diabetes mellitus (T2D) compared with age, gender, and sex-matched control subjects. The frequency of cells expressing the monocyte/macrophage marker CD14 was decreased in patients with T2D compared with control subjects. The frequency of cells expressing the M1 macrophage marker CD80 (pro-inflammatory phenotype) was increased whereas the frequency of cells expressing the M2 macrophage marker CD163 (anti-inflammatory phenotype) was decreased in patients with T2D compared with control subjects. Values are mean ± SEM. Statistical comparisons were conducted with the Student's *t*-test.

CTLA-4 = cytotoxic T-lymphocyte associated protein 4; IgG = immunoglobulin G.

high (R3) SSC as shown in Figure 1B. Importantly, the frequency of cells with ALDH^{hi}SSC^{low} (progenitor cells) and ALDH^{hi}SSC^{mid} (primarily monocytes) phenotypes were equal in patients with T2D compared with control subjects (Figures 1C and 1D). In contrast, cells with the ALDH^{hi}SSC^{hi} (granulocytes) phenotype were >2-fold increased in patients with T2D. These ALDH^{hi}SSC^{hi} cells expressed neutrophil markers, including CD15, CD16b, and CD66b; some were positive for the monocyte marker CD14, and all cells were negative for CD34 co-expression. These findings confirm that the ALDH^{hi}SSC^{hi} population primarily comprised granulocytes that can propagate inflammatory processes, again marking increased circulating inflammatory cell content in patients with T2D.

ALDH^{hi}SSC^{low} CELLS WITH PROGENITOR CELL SURFACE MARKER CO-EXPRESSION WERE DECREASED IN PATIENTS WITH T2D. ALDH^{hi}SSC^{low} cells can be described as a heterogeneous progenitor cell population comprising primarily hematopoietic (>90%) and endothelial (<10%) cell lineages (17), and they have both been shown to support angiogenic blood vessel formation in immunodeficient mice with femoral artery ligation. Although these cells are extremely rare in the peripheral circulation (<0.1%), they possess a robust proangiogenic signaling profile and also contain rare endothelial precursor cells with the capacity to integrate into sprouting vessels (35–38). Thus, detection of circulating ALDH^{hi}SSC^{low} cells is critical for the assessment of provascular regenerative capacity. Although we detected no significant differences in the overall frequency of ALDH^{hi}SSC^{low} or ALDH^{hi}SSC^{mid} cells between patients with T2D and matched control subjects, ALDH^{hi}SSC^{low} cells were further assessed for primitive hematopoietic and endothelial cell surface expression (CD34+/CD133+), early myeloid cell surface marker expression (CD34+/CD33+), and primitive migratory progenitor cells (CD34+/CXCR4+ cells). Both proangiogenic hematopoietic and vessel-integrating endothelial progenitor cells commonly express CD34 (36). The frequency of cells expressing CD34 alone was not different in the ALDH^{hi}SSC^{low} population (Supplemental Table 2) but patients with T2D exhibited significantly decreased frequencies of primitive progenitor (Figures 2A to 2C), early myeloid (Figures 2D to 2F), and migratory progenitor (Figures 2G to 2I) cells compared with control subjects; these findings indicate reduced vascular regenerative progenitor cell representation in patients with T2D (23,39).

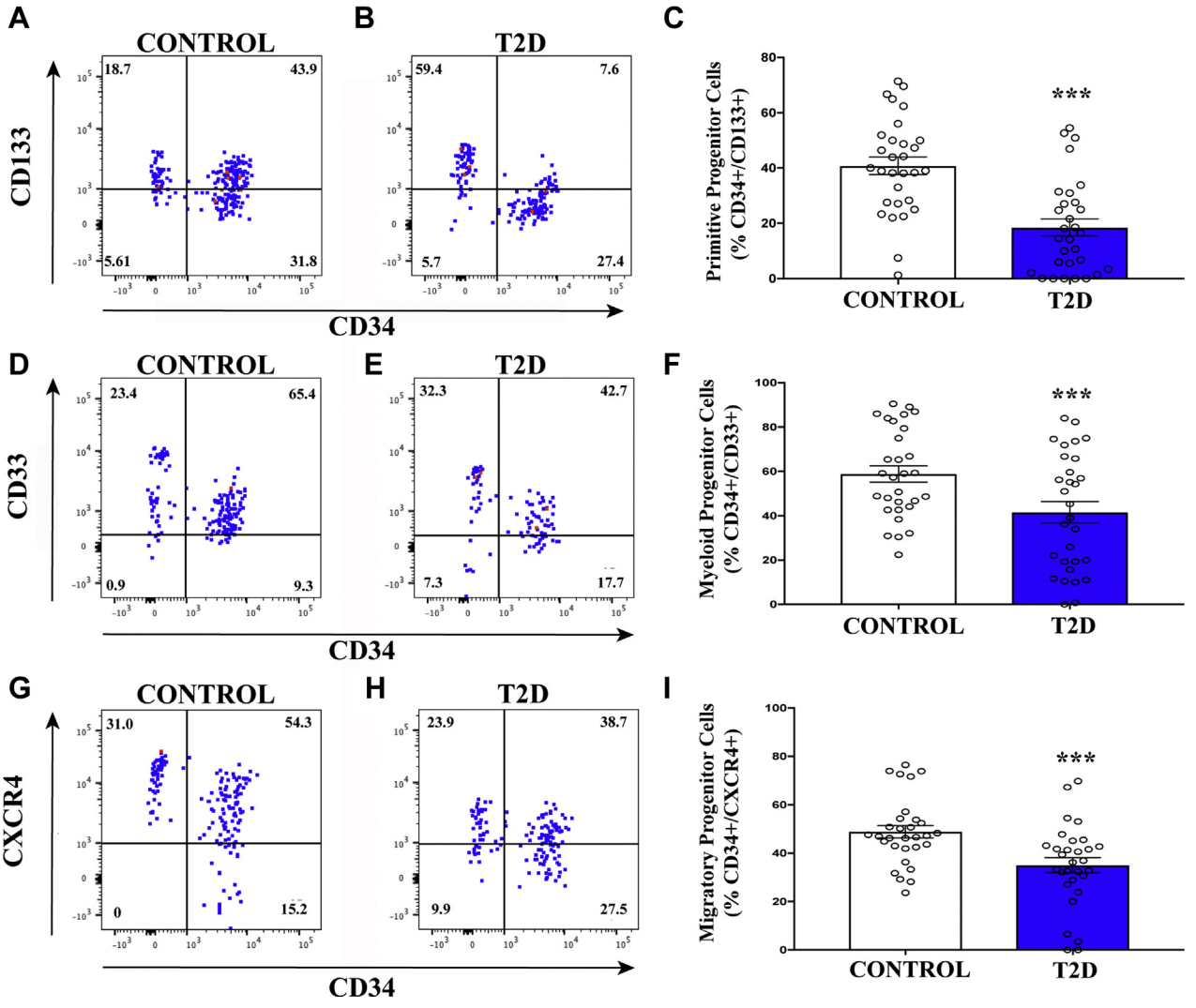
FIGURE 1 **Circulating Progenitor Cell Subpopulations Are Discerned According to High ALDH-Activity and SSC Properties**



(A and B) Representative flow cytometry plots using *N,N*-diethylaminobenzaldehyde (DEAB) to inhibit aldehyde dehydrogenase (ALDH) activity establishing gates for low versus high ALDH-activity. Without inhibition, cells with high ALDH-activity exhibit increased fluorescence intensity (right shift) and detect primitive cells with a self-protective progenitor cell phenotype. High ALDH-activity combined with side scatter (SSC) properties selects for a progenitor cell subpopulation with low intracellular complexity (R1 = ALDH^{hi}SSC^{low} cells), a monocyte subpopulation with intermediate intracellular complexity (R2 = ALDH^{hi}SSC^{mid} cells), and a granulocytic subpopulation with high intracellular complexity (R3 = ALDH^{hi}SSC^{hi} cells). **(C and D)** Representative flow cytometry plots showing the frequencies of each population in control subjects and patients with type 2 diabetes mellitus (T2D). Compared with control subjects, patients with T2D exhibited an increased frequency of ALDH^{hi} cells within the granulocyte subpopulation (R3) and an equal frequency of circulating ALDH^{hi} cells with low (R1) and intermediate (R2) complexity. Values are mean ± SEM. ***p < 0.001 with the Student's *t*-test.

ALDH^{hi}SSC^{low} CELLS WITH ENDOTHELIAL-ASSOCIATED PHENOTYPES WERE DECREASED IN PATIENTS WITH T2D. We next compared the ALDH^{hi}SSC^{low} population for the expression of endothelial cell-associated and pericyte-associated adhesion molecules. Although the frequency of cells co-expressing CD34 and platelet endothelial cell adhesion molecule-1 (CD31) was equivalent between patients with T2D and control subjects (Figures 3A to 3C), primitive (CD34+) cells expressing vascular endothelial-cadherin and the pericyte marker CD146 were decreased in patients with T2D compared with control subjects

FIGURE 2 Circulating ALDH^{hi}SSC^{low} Cells With Primitive, Myeloid, and Migratory Phenotypes Are Decreased in Patients with T2D

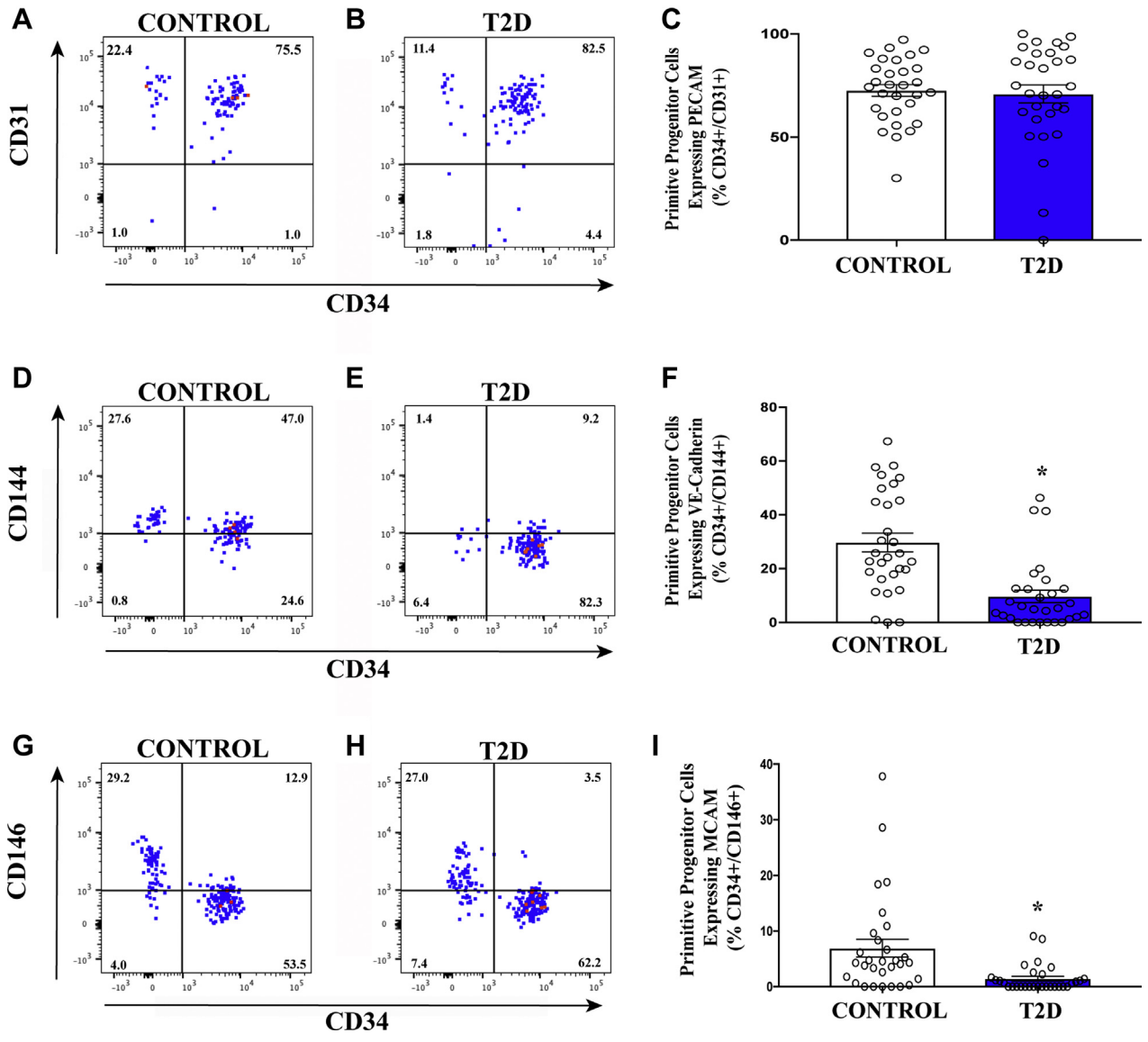


(A–C) The frequency of circulating ALDH^{hi}SSC^{low} progenitor cells with primitive cell phenotype (CD34+CD133+) was reduced in patients with T2D compared with control subjects. **(D–F)** The frequency of circulating ALDH^{hi}SSC^{low} progenitor cells with early myeloid cell phenotype (CD34+CD33+) was reduced in patients with T2D compared with control subjects. **(G–I)** The frequency of circulating ALDH^{hi}SSC^{low} progenitor cells with migratory phenotype (CD34+CXCR4+) was reduced in patients with T2D compared with control subjects. Values are mean ± SEM. ***$p < 0.01$ with the Student's *t*-test. Abbreviations as in Figure 1.

(Figures 3D to 3I). Cells expressing these markers are required for vasculogenic vessel formation (40). These findings were also consistent with the depletion of proangiogenic circulating cell content, potentially leading to a dysfunctional vascular regenerative response in patients with T2D.

ALDH^{hi}SSC^{mid} CELLS WITH PRIMITIVE AND M2 PHENOTYPES WERE DECREASED IN PATIENTS WITH T2D. Primitive circulating monocytes with proinflammatory and anti-inflammatory cytokine secretion patterns (33,41,42) were further assessed by analyses of CD68+ cells co-expressing CD34 or CD80 versus CD163 (M1/M2 marker) specifically within the ALDH^{hi}SSC^{mid} cell subset. These cells can best be described as monocytes that possess either anti-inflammatory or proinflammatory secretory activities characterized by co-expression of M1/M2 polarization markers, respectively (33,43). M2-polarized monocytes and tissue-resident M2 macrophages can contribute toward arteriogenic processes through secretion of cytokines and metalloproteinases that remodel pre-existing collateral vessels (44). In

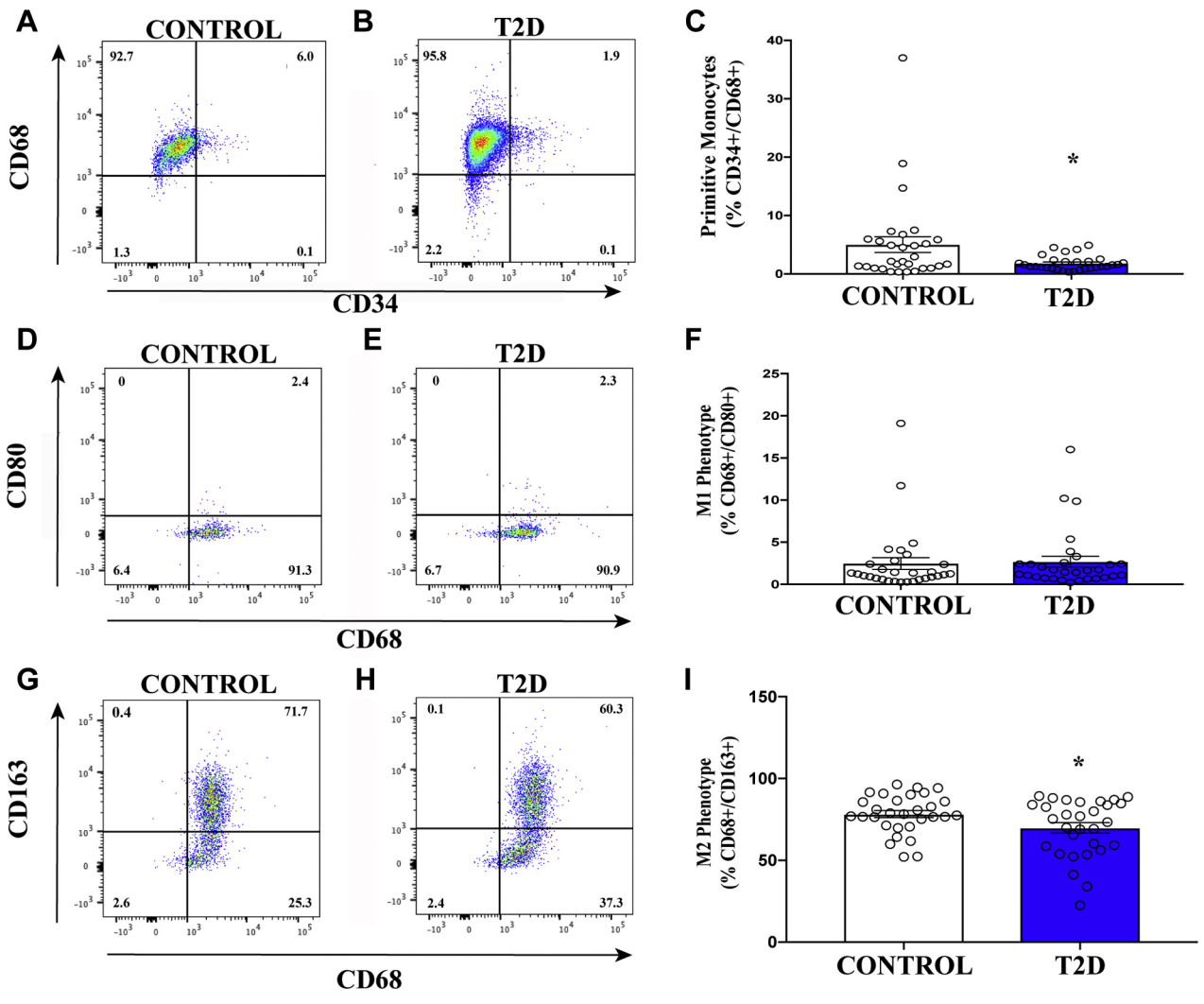
FIGURE 3 Circulating ALDH^{hi}SSC^{low} Cells With Endothelial Cell and Pericyte-associated Adhesive Phenotypes Are Decreased in Patients With T2D



(A-C) The frequency of circulating ALDH^{hi}SSC^{low} progenitor cells co-expressing CD34 with hematopoietic/endothelial cell marker platelet endothelial cell adhesion molecule (PECAM) (CD31) was equivalent in patients with T2D compared with control subjects. **(D-F)** The frequency of circulating ALDH^{hi}SSC^{low} cells in progenitor cells co-expressing CD34 with the endothelial cell-associated marker vascular endothelial (VE)-cadherin (CD144) was decreased in patients with T2D compared with control subjects. **(G-I)** The frequency of circulating ALDH^{hi}SSC^{low} progenitor cells co-expressing CD34 with the endothelial/pericytes marker melanoma cell adhesion molecule (MCAM) (CD146) was decreased in patients with T2D compared with control subjects. Values are mean ± SEM. *$p < 0.001$ with the Student's *t*-test. Other abbreviations as in Figure 1.

contrast, M1 macrophages generally contribute toward inflammatory processes that may impede new vessel progression (18,43,45). Although we detected no significant differences in the frequency of ALDH^{hi}SSC^{mid} cells with M1 (CD68+/CD80+) phenotypes (Figures 4D to 4F), patients with T2D exhibited significantly reduced frequency of ALDH^{hi}SSC^{mid} cells with primitive (CD34+/CD68+) (Figure 4A to 4C) or M2 (CD34+/CD163+) (Figures 4G to 4I) phenotypes compared with control subjects. These findings validated the reduction in circulating monocytes with M2 polarization phenotype shown in Table 1 and suggest that patients with T2D may also exhibit reduced capacity to mediate arteriogenic vessel remodeling.

FIGURE 4 Circulating ALDH^{hi}SSC^{mid} Cells With M2 Phenotype Are Decreased in Patients With T2D

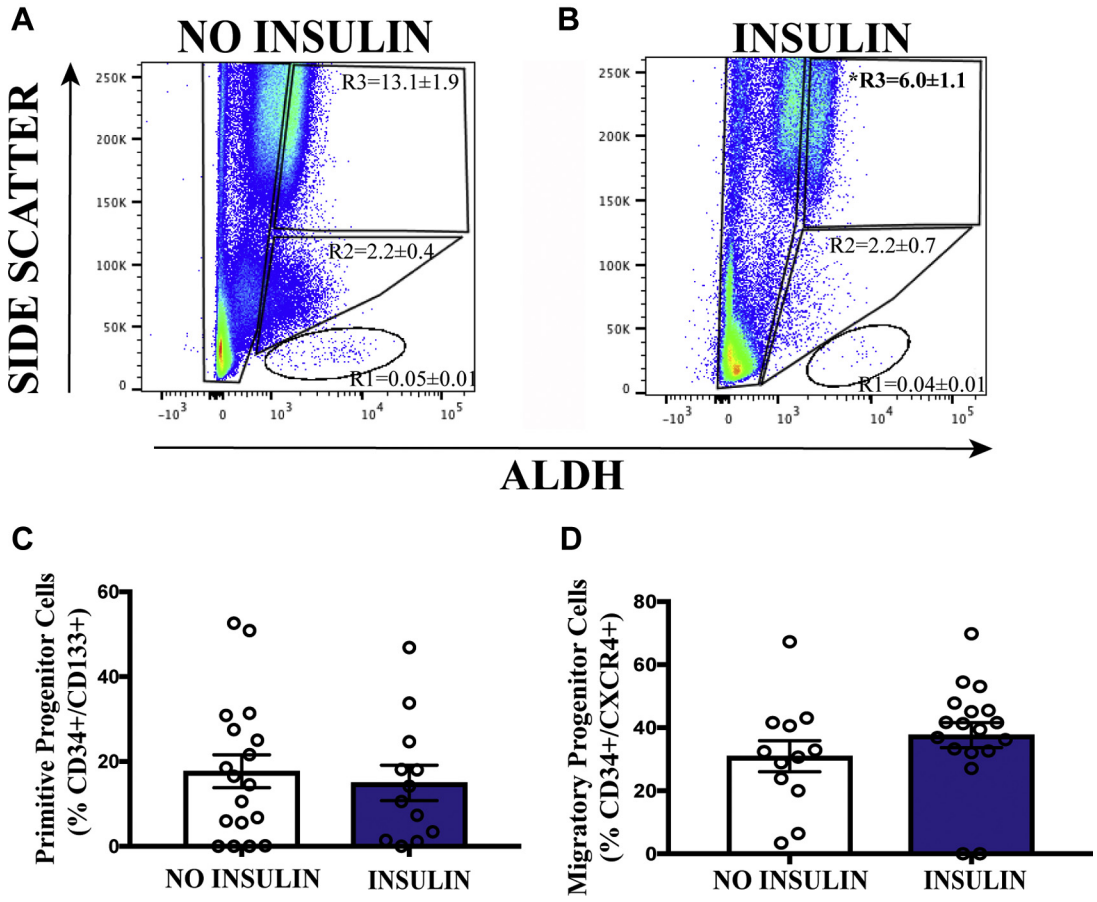


(A to C) The frequency of circulating ALDH^{hi}SSC^{mid} cells co-expressing CD34 with the macrophage scavenger receptor (CD68) was decreased in patients with T2D compared with control subjects. **(D to F)** The frequency of circulating ALDH^{hi}SSC^{mid} cells co-expressing CD68 with the M1 macrophage–associated marker CD80 was equal in patients with T2D compared with control subjects. **(G to I)** The frequency of circulating ALDH^{hi}SSC^{mid} progenitor cells co-expressing CD68 with the M2 macrophage–associated marker CD163 was decreased in patients with T2D compared with control subjects. Values are mean ± SEM. *$p < 0.05$ with the Student's *t*-test. Other abbreviations as in Figure 1.

INSULIN ADMINISTRATION CORRELATED WITH REDUCED CIRCULATING GRANULOCYTE FREQUENCY. To determine whether factors such as sex, insulin use, or HbA_{1c} status played a correlative role in the frequency of circulating provascular progenitor cells in patients with T2D, these patients were divided into 2 groups based on the median value for each category. The frequency of cell subsets with high ALDH-activity with primitive (CD34+/CD133+) and migratory (CD34+/CXCR4+) cell surface phenotype was equivalent in male (n = 12) and female (n = 18) patients with T2D (Supplemental Figure 2). Surprisingly, patients with HbA_{1c} values ≤7.0% (n = 15) or >7.0% (n = 15) also showed no significant difference in the frequencies of ALDH^{hi} cell subpopulations or in ALDH^{hi}SSC^{low} progenitor cells that expressed primitive or migratory phenotypes (Supplemental Figure 3). These data suggest that higher HbA_{1c} levels did not correlate with a reduction in circulating progenitor cell content during T2D.

We next segregated patients according to their use of daily insulin injections, a general indication of

FIGURE 5 Circulating ALDH^{hi}SSC^{hi} Inflammatory Cells Are Decreased in Patients Taking Insulin

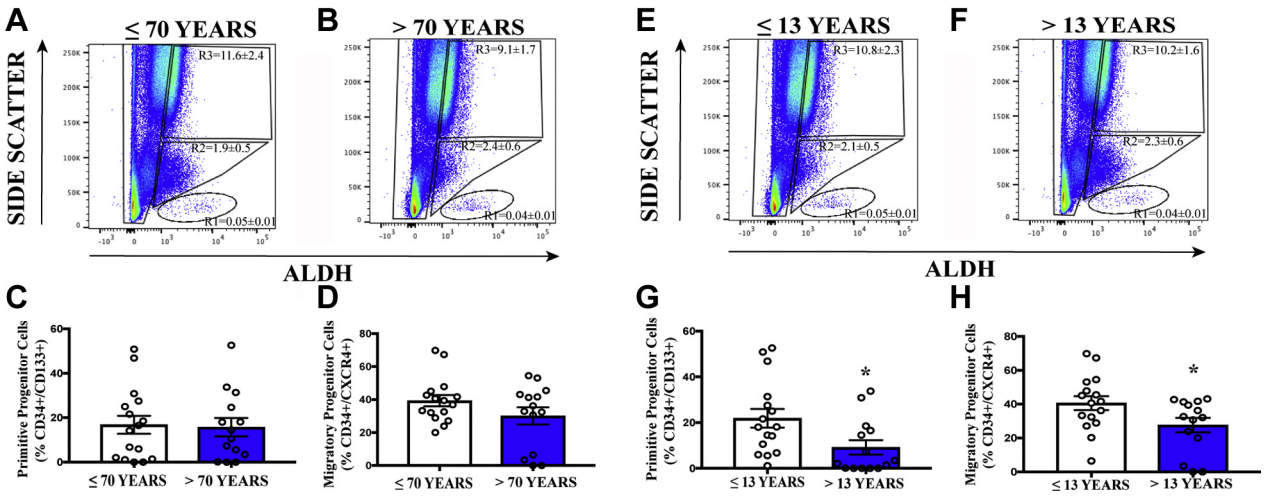


(A and B) The frequency of cells with high ALDH-activity and high SSC properties was decreased in patients with T2D taking insulin. **(C and D)** The frequency of primitive and migratory progenitor cells was equivalent in patients with T2D despite insulin therapy. Values are mean ± SEM. *p < 0.05 with the Student's *t*-test. Abbreviations as in Figure 1.

more advanced T2D whereby glycemia is not controlled by diet, exercise, and medication. There was a significant decrease in the frequency of circulating pro-inflammatory ALDH^{hi}SSC^{hi} granulocytes in patients receiving insulin therapy (Figures 5A and 5B). These data suggested reduced inflammation in patients who received insulin therapy; however, circulating primitive progenitor cell frequencies were not affected by insulin use (Figures 5C and 5D).

PROVASCULAR PROGENITOR CELL EXHAUSTION CORRELATED WITH INCREASED DURATION OF T2D. To further assess regenerative cell exhaustion during T2D, a process through which stem and progenitor cell frequency is reduced due to chronic disease, patients with T2D were subdivided based on chronological age or duration of T2D. Although increased age (≤70 years, n = 14; >70 years, n = 16) did not alter ALDH expression levels (Figures 6A and 6B) or primitive progenitor (CD34+/CD133+) or migratory (CD34+/CXCR4+) cell surface marker expression (Figures 6C and 6D), patients with increased duration of T2D (≤13 years, n = 15; >13 years, n = 15) demonstrated no difference in ALDH expression levels (Figure 6E and 6F), but did, however, exhibit significantly decreased frequency of primitive (CD34+/CD133+) and migratory (CD34+/CXCR4+) progenitor cells (Figures 6G and 6H). These data suggested that patients with prolonged T2D duration was the only analyzed subgroup that exhibited significantly reduced circulating proangiogenic progenitor cell content (23,46–48).

FIGURE 6 Circulating ALDH^{hi}SSC^{low} Cells With Primitive and Migratory Progenitor Cell Phenotypes Decreased With Longer Duration of Diabetes



(A and B) In patients with T2D, the frequency of cells with high ALDH-activity was equivalent in patients ≤70 years of age compared with patients >70 years of age with T2D. **(C and D)** The frequency of circulating primitive progenitor cells (CD34+/CD133+) and migratory progenitor cells (CD34+/CXCR4+) was equivalent in patients ≤70 years of age compared with patients >70 years of age. **(E and F)** In patients with T2D, the frequency of cells with high ALDH-activity was equivalent in patients with diabetes duration ≤13 years compared with patients with diabetes duration >13 years. **(G and H)** However, the frequency of circulating primitive progenitor cells (CD34+/CD133+) and migratory progenitor cells was decreased in patients with diabetes duration ≤13 years compared with patients with diabetes duration >13 years. Values are mean ± SEM. *p < 0.05 with the Student's *t*-test. Abbreviations as in Figure 1.

DISCUSSION

The current study presents a novel diagnostic flow cytometry assay, using high ALDH-activity, a functional measure for a conserved progenitor cell function, combined with selected primitive and mature cell surface marker analyses, to characterize the frequency of cellular subsets with proangiogenic versus proinflammatory phenotypes from the peripheral blood of human patients with T2D compared with individuals without diabetes. The use of cell surface markers independently (e.g., CD34, CD133), as previously reported in several studies (35,39,49), revealed few differences in circulating cell frequencies between groups. However, by first detecting cells with high ALDH-activity, combined with SSC properties to discern granulocytic/neutrophil (SSC^{hi} cells), monocyte (SSC^{mid} cells), or primitive progenitor cell (SSC^{low} cells) subpopulations, allowed for additional comparison of primitive, progenitor cell markers (CD34, CD133) previously associated with proangiogenic secretory functions (37,38,50). By using this combined functional and phenotypic strategy, patients with T2D consistently exhibited the following unique characteristics: 1) an increased frequency of ALDH^{hi}SSC^{hi} granulocytes predicted to propagate inflammatory burden (51,52); 2) a reduced frequency of circulating ALDH^{hi}SSC^{mid} monocytes with CD14+ co-expression (33,43); 3) a shift in ALDH^{hi}SSC^{mid} cell M1/M2 balance toward the pro-inflammatory M1 phenotype (31,53); and 4) a decreased frequency of rare circulating ALDH^{hi}SSC^{low} progenitor cells that co-expressed CD34 and primitive (CD133), early myeloid (CD33), migratory (CXCR4), endothelial adhesion (CD144), or pericyte (CD146) cell surface markers. Collectively, these data suggest that a prolonged duration of T2D promotes a pro-inflammatory milieu, affecting both granulocytes and monocytes, in addition to depletion of rare progenitor cells shown previously to coordinate proangiogenic blood vessel repair in animal models (23,35,48,54,55).

The overall frequency of ALDH^{hi}SSC^{low} progenitor cells or CD34-expressing cells was surprisingly not decreased in patients with T2D compared with the control subjects. Selection for cells with high ALDH-activity (4,56) or CD34 expression (30,32,57,58) has been used in clinical trials as highly purified cell populations transplanted from autologous BM to combat ischemic disease. In contrast, a significant reduction in the frequency of circulating monocytes (33,42,43) with anti-inflammatory M2 phenotype (31,59,60) was easily detected in patients with T2D. These observations may be attributed to differences in the relative frequencies of circulating cells in the

peripheral blood of patients with T2D and nondiabetic control subjects. The frequency of ALDH^{hi}SSC^{low} cells in the peripheral blood of both cohorts was exceedingly low, comprising <0.1% of peripheral blood mononuclear cells, whereas CD14+ monocytes were >100-fold more abundant. Thus, careful analyses of the rare ALDH^{hi}SSC^{low} cell subset required multicolor assessment of CD34 co-expression in addition to multiple cell surface molecules with functional significance to quantify the depletion of circulating proangiogenic progenitor cells. The expression of primitive (CD133), early myeloid (CD33), chemokine (CXCR4), and cellular adhesion (CD144) molecules was consistently reduced in patients with T2D compared with nondiabetic control subjects. These findings suggest that ALDH^{hi} progenitor cells in the circulation of patients with T2D may exhibit deficits in cell adhesion and migration capacity toward ischemic endothelium. Therefore, direct comparison of ALDH^{hi}SSC^{low} cells for colony formation (24,25,34), cytokine secretion patterns (24), and migratory function in patients with T2D and control subjects are next required to assess potential functional deficits in circulating provascular cell populations.

Generalized inflammatory excess combined with circulating provascular progenitor cell depletion may contribute to an underlying issue affecting adult hematopoietic and endothelial progenitor cell maintenance in patients with T2D. This phenomenon, termed regenerative cell exhaustion, documents the loss of vascular regenerative capacity due to premature progenitor cell maturation and a reduction in the number of undifferentiated cells within the BM reservoir (27,28,46,61,62). Increased inflammation associated with chronic T2D is also known to induce increased expression of NADPH oxidase-1, which regulates the formation of ROS (28,63,64). Conceptually, although progenitor cells possess defense mechanisms such as elevated ALDH-activity to reduce oxidative stress and prevent premature apoptosis, excessive ROS may contribute to aberrant differentiation and maturation regulation within progenitor cells, resulting in the premature departure of vascular regenerative precursors from the endosteal niche in the BM (64–66). In the peripheral circulation, without the influence of developmental factors (e.g., Wnt [wingless related integration site], Notch) in the BM stem cell niche (67–70), cells are expected to demonstrate aberrant differentiation, generating dysfunctional cells with reduced contribution toward blood vessel repair and regeneration (71,72). Thus, further measurements of cell frequencies using this approach in the BM and other tissues may help correlate circulating cell deficiencies with compromised function in tissues.

To further show the utility of this assay, we stratified the T2D cohort based on patient sex, age, duration of diagnosed T2D, HbA_{1c} value, and the requirement for insulin. Notably, only the duration of T2D correlated with reduced proangiogenic progenitor cell frequency. Indeed, regenerative cell depletion became more prominent with extended duration of T2D.

To our knowledge, the current study is the first to clearly document the depletion of circulating provascular progenitor cell content by using ALDH-activity during established T2D in human subjects. Furthermore, patients with T2D exhibited a departure from an anti-inflammatory M2 phenotype toward a pro-inflammatory M1 phenotype compounded by an increase in circulating granulocytes. Throughout these analyses, we documented changes in the frequency of circulating cell phenotypes implicated in the restoration of vascular regenerative function in patients with T2D. In addition, this research provides a starting point for development of novel therapeutic approaches to combat ischemic vascular disease progression during T2D. It is evident that regenerative cell depletion and heightened inflammation during T2D generates a harsh microenvironment for functional revascularization (23,27,48,62,63). By reducing inflammation and limiting ROS, development of therapeutic strategies tailored to the restoration of the vascular regenerative cell generation and function may aid in the prevention of ischemic vascular comorbidities that are so devastating during the progression of T2D.

STUDY LIMITATIONS AND FUTURE DIRECTIONS. Care must be taken when extending the utility of these studies toward potential clinical application. First, the detection of very rare circulating cell populations by using flow cytometry provides a diagnostic tool to measure altered cell frequencies during T2D. Future long-term clinical studies should incorporate multiple assessments of circulating cell subpopulations as T2D progresses. With mindful trial design, this assay may reveal the sensitivity required to correlate changes in circulating cell frequencies with specific outcomes such as adverse cardiovascular events. Second, circulating cell subpopulations were not assayed for relevant proangiogenic function in this study. In future studies, we intend to assess colony formation as well as secretory and migratory functions of relevant cell populations as T2D

progresses. Third, analyses of tissue-resident progenitor cell or macrophage frequencies were not conducted in this study and are needed to determine how circulating cell content correlates with cell frequencies in the BM or other tissues affected by ischemia such as the heart or skeletal muscle. Finally, disease comorbidities such as obesity, atherosclerotic burden, previous ischemic events, and drug use need to be carefully controlled between groups when interpreting the relevance of these measurements on the potential alteration of T2D progression.

CONCLUSIONS

Circulating cells with pro-inflammatory phenotype were more abundant in patients with T2D, and rare ALDH-expressing progenitor cells previously associated with vascular regenerative function were markedly reduced. In addition, adhesive and migratory cell surface marker co-expression associated with homing to areas of ischemia and secretion of proangiogenic effectors was deficient on circulating ALDH^{hi} progenitor cells in patients with T2D. Collectively, alterations in these circulating cell phenotypes may presumably contribute to the gradual loss of the capacity for vascular repair. Although we can clearly detect differences in the frequencies of rare circulating progenitor cells by using high ALDH-activity, further studies are warranted to assess the vascular regenerative functions of these circulating cell populations. Functional analyses relevant to ischemic disease include the formation of myeloid hematopoietic and endothelial cell colonies, tubule formation, migration to areas of ischemia, and secretion of proangiogenic cytokines that coordinate vascular regenerative processes. Furthermore, functional testing of the capacity of these cell types to contribute to perfused neovessel formation in vivo is still required. Nonetheless, potential reversal of this "exhausted" vascular regenerative cell phenotype during T2D, through regenerative medicine strategies or by administration of therapeutic agents with documented cardiovascular protective effects, represents an exciting avenue to be tested in future studies.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Individuals with T2D are at a heightened risk of developing cardiovascular disorders and often endure poor outcomes after a cardiovascular event. This study shows, for the first time, that compared with individuals who are normoglycemic, those with established T2D exhibit depleted circulating vascular regenerative progenitor cell content measured in the circulation by using ALDH-activity, a conserved protective function demonstrated by proangiogenic endothelial and hematopoietic progenitor cells. In contrast, circulating monocytes exhibit a migration from a protective anti-inflammatory phenotype to one that is proinflammatory, collectively discouraging functional revascularization.

TRANSLATIONAL OUTLOOK: Using a combination of ALDH-activity measurement and cell surface marker expression, we have developed a novel diagnostic flow cytometry assay to evaluate the balance between circulating proangiogenic progenitor and proinflammatory cell content in peripheral blood. This study provides a critical translational perspective by the suggestion that the balance in these cells during the progression of T2D is critical to the development of and recovery from ischemic vascular comorbidities. The potential of developing this assay into a diagnostic tool to estimate the capacity to mitigate ischemia via a provascular regenerative response represents an exciting avenue for exploration and will need to be investigated.

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APPENDIX For supplemental figures and tables, please see the online version of this paper.