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The number of circulating CD26 expressing cells is decreased in critical COVID-19 illness

Joanne T. deKay[1] | Teresa L. May[1,2] | Richard R. Riker[2] | Jonathan Rud[2] | David J. Gagnon[1,3,4] | Douglas B. Sawyer[1,2] | David B. Seder[1,2] | Sergey Ryzhov[1]

[1]Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, Maine, USA

[2]Department of Critical Care Services, Maine Medical Center, Portland, Maine, USA

[3]Department of Pharmacy, Maine Medical Center, Portland, Maine, USA

[4]Tufts University School of Medicine, Boston, Massachusetts, USA

Correspondence
Sergey Ryzhov, Maine Medical Center Research Institute, 81 Research Drive, Scarborough ME 04074, USA.
Email: sryzhov@mmc.org
David B. Seder, Maine Medical Center Department of Critical Care Services, 22 Bramhall St, Portland, ME 04102, USA.
Email: sederd@mmc.org

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Abstract

We evaluated the number of CD26 expressing cells in peripheral blood of patients with COVID-19 within 72 h of admission and on day 4 and day 7 after enrollment. The majority of CD26 expressing cells were presented by $CD3^{+}CD4^{+}$ lymphocytes. A low number of CD26 expressing cells were found to be associated with critical-severity COVID-19 disease. Conversely, increasing numbers of CD26 expressing T cells over the first week of standard treatment was associated with good outcomes. Clinically, the number of circulating CD26 cells might be a marker of recovery or the therapeutic efficacy of anti-COVID-19 treatment. New therapies aimed at preserving and increasing the level of CD26 expressing T cells may prove useful in the treatment of COVID-19 disease.

KEYWORDS
CD26, COVID-19, DPP4, immune response, lymphocytes

1 | INTRODUCTION

The role of CD26/dipeptidyl peptidase IV (DPP IV) in COVID-19 has been intensively discussed [1, 2], primarily as a potential entry receptor for SARS-CoV-2 [3]. CD26 is ubiquitous in many tissues and cell types, including T cells characterized by high cell-surface CD26 expression [4]. Given frequent natural migration between different lymphoid and nonlymphoid tissues, lymphocytes that express CD26 could be involved in virus progression throughout the body. However, whether or not CD26 expressing cells are associated with the severity or outcomes of COVID-19 have not been reported previously.

CD26 is a multifunctional glycoprotein [5]. Beside the dipeptidyl aminopeptidase activity [6], CD26 induces intracellular signaling associated with the development and activation of immune cells, including effector T cells [7]. In addition, CD26 is an adenosine deaminase anchoring protein involved in the regulation of the induction and

resolution of inflammation via purinergic signaling modulation [8]. In this study, we characterized T cell subpopulations, and specifically CD26 expressing cells, in patients with COVID-19 and healthy donors to identify associations between the number of T cells and the severity, clinical features, and outcomes of COVID-19.

2 | MATERIALS AND METHODS

2.1 | Patients

The study and informed consent process were approved by the Maine Medical Center Institutional Review Board. Informed consent was obtained from the patient or the legally authorized representative (LAR) using secure electronic consent to prevent disease transmission. Patients were enrolled between July and December 2020 within 72 h of being hospitalized with PCR-confirmed SARS-CoV-2 infection (real-time RT-PCR test, NorDx Laboratories, Portland, ME). We excluded patients who were under 18 years of age, represent a vulnerable population, had a hemoglobin <8 g/dl, or from whom consent was not obtained.

COVID-19 disease severity was defined using clinical criteria from the US Centers for Disease Control and Prevention(CDC) [9]: (1) mild illness: any signs and symptoms of COVID-19 (e.g., fever, cough, sore throat, malaise, headache, muscle pain) without shortness of breath, dyspnea, or abnormal chest imaging, (2) severe illness: respiratory rate > 30 breaths per minute, SpO_2 < 94% on room air at sea level (or, for patients with chronic hypoxemia, a decrease from baseline of >3%), a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) <300 mmHg, or lung infiltrates >50%, and (3) critical illness: respiratory failure, septic shock, and/or multiple organ dysfunction.

Peripheral blood samples were obtained by the Maine Medical Center BioBank from asymptomatic and SARS-CoV-2 negative subjects as a control population. Pertinent demographic and clinical data were collected from the electronic medical record for all study participants.

2.2 | Standards of care

The standard of care during hospitalization of patients with COVID-19 evolved during the study period as new information became available. It often included the antiviral drug remdesivir and synthetic adrenocortical glucocorticoid dexamethasone; many received systemic anticoagulation, and antibiotics for community-acquired pneumonia until it was determined they did not have concurrent bacterial infection. IL-1 or IL-6 inhibitors were not administered to these patients. Our COVID-19 cohort included a few asymptomatic patients admitted for other reasons who were determined to have SARS-CoV-2 infection on administrative testing, although most had severe or critical illness as described above. Mechanically ventilated patients received lung-protective ventilation, proning for refractory hypoxemia, and standard critical care therapies (e.g., neuromuscular blocking agents, sedation, and analgesia). Patients may also have received renal replacement therapy (i.e., intermittent hemodialysis or continuous veno-venous hemofiltration) or arteriovenous or venovenous extracorporeal membrane oxygenation, as indicated.

2.3 | Blood sample collection

Subjects underwent phlebotomy on the day of enrollment and, when feasible, at day 4 and day 7 post-enrollment. Blood (8.5 ml) was collected from COVID-19 and control subjects using BD Vacutainer ACD tubes.

Platelet-free plasma was prepared at room temperature using 2-step centrifugation, each at 2000 x g for 20 min. After processing, plasma was stored at −80°C until further analysis. No more than one freeze/thaw cycle was allowed for PFP samples to prevent protein degradation.

2.4 | Flow cytometric analysis

Blood aliquots (50 μl) for flow cytometric analysis underwent erythrocyte lysis with ammonium chloride lysing solution (150 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$, and 1 mmol/L EDTA, pH 7.4). White blood cells (WBC, 10^6 cells/ml) were treated with whole molecule mouse and human IgGs to prevent nonspecific binding, followed by incubation with relevant antibodies for 25 min at 4°C. Cells were washed once with 10 volumes of cold PBS/0.5% BSA/2 mM EDTA before data acquisition.

Subpopulations of WBC were analyzed using the following antibodies: FITC conjugated CD3 (UCHT1), APC/Cy7-CD4 (OKT4), PeCy7-CD8a (HIT8a), PeCy7-CD14 (HCD14), APC-Cy7-CD16 (3G8), APC-CD26 (BA5b), PE-CD45 (HI30), PE-CD73 (AD2), APC-HLA-DR (LN2430) (all purchased from BioLegend).

An aliquot of blood was used to determine the absolute number of $CD45^{pos}$ WBC using TruCount™ Tubes (BD Biosciences) according to the manufacturer's protocol. The absolute values for cell subpopulations, including CD26 expressing cells, were calculated after multiplying the total number of WBC to the percent of events in the corresponding gate. Viable and nonviable cells were distinguished using 4′,6-diamidino-2-phenylindole (DAPI) at the final concentration of 20 ng/ml (72.5 nM). Data acquisition was performed on a MacsQuant Analyzer 10 (Miltenyi Biotec, Inc.); data were analyzed using WinList 5.0 and FlowJo 10.7 software.

2.5 | Analysis of circulating IL-6, IL-8, CXCL2, MCP-1, CCL4, and CCL23

Levels of interleukin-6 (IL-6), interleukin-8 (IL-8), C-X-C motif chemokine ligand 2 (CXCL2), monocyte chemoattractant protein-1 (MCP-1), C–C motif chemokine ligand 4 (CCL4), and C–C motif chemokine

ligand 23 (CCL23) in platelet-free plasma were measured using ELISA kits (Bio-techne/R&D Systems).

2.6 | Statistical analysis

Data are expressed as mean value and standard error for normal distribution or as median and interquartile range if the distribution is skewed. Comparisons between two groups were performed using Mann–Whitney tests. Comparisons between more than two groups were performed using Kruskal-Wallis test with Dunn's multiple-comparisons test, or two-way ANOVA with Tukey multiple comparisons test. Correlation analysis was performed using a Spearman (skewed distribution) correlation. Statistical analyses were performed with GraphPad Prism 7.05. p <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Study subjects

A convenience sample of 29 patients with COVID-19 and 12 control subjects were analyzed. There were no differences between the study subjects and control subjects in terms of age, sex, and body mass index (Table 1).

TABLE 1 Characteristics of control subjects and subjects with COVID-19

Characteristic	Control subjects ($n = 12$)	Subjects with COVID-19 ($n = 29$)
Age, mean ± SD	67 ± 9.6	63.8 ± 14.7
Male, *n* (%)	8 (66.7)	19 (65.5)
Race, (%)	White (100)	White (100)
BMI, mean ± SD	28.2 ± 5	32 ± 7
COVID severity[a], # (%)		
Mild, severe, critical		7, 10, 12 (24, 35, 41)
Estimated FiO_2 (%), median (IQR)		32, (24, 50)
Days since symptom onset, mean ± SD		7.7 ± 4.8
Antibiotics, # (%)		18 (62.1)
Steroids, # (%)		23 (79.3)
Hemodialysis, # (%)		3 (10.3)
Thrombosis, # (%)		6 (20.7)
Hospital LOS, median (IQR)		8 (4, 16)
Discharge survival, # (%)		24 (82.8)

Abbreviations: FiO_2, fraction of inspired oxygen; IQR, interquartile range; LOS, length of stay.
[a]Mild: no need for supplemental oxygen; Severe: supplemental oxygen required; Critical: critically ill with respiratory failure.

3.2 | The majority of CD26 expressing cells are CD3/CD4 T lymphocytes

We examined the immune cell phenotype by flow cytometry. Freshly isolated white blood cells were stained with specific antibodies, and major subpopulations of viable, singlet white blood cells (after exclusion of unlysed erythrocytes) (Figure 1A–C) were analyzed for the CD26- and CD73-expressing cells ($CD26^{pos}$ and $CD73^{pos}$), and subsets of CD3/CD4 cells within $CD26^{pos}$ and $CD26^{pos}CD73^{pos}$ populations in groups of patients with COVID-19 (Figure 1D–F) and control subjects (Figure 1G–I). CD73 is expressed on immunosuppressive T cells [10, 11] as opposed to CD26, which is highly expressed on Th1 and Th17 T effector cells [12]. The analysis revealed that the majority of $CD26^{pos}$ cells are represented by $CD3^{+}CD4^{+}$ T lymphocytes in both groups. The majority of CD73 expressing cells were B lymphocytes (data not shown).

3.3 | The number of CD26 expressing cells inversely correlated with the severity of COVID-19 illness and survival

To determine the relationship between CD26 expressing cells and other measured clinical and laboratory parameters, we performed correlation analysis in the group of patients with COVID-19 (Figure 2A). Inverse correlations were identified between the number of CD26 expressing cells as well as $CD3^{+}$, $CD3^{+}CD4^{+}$, and $CD3^{+}CD4^{+}CD26^{+}$ T lymphocyte subsets and the severity of COVID-19 illness and the level of creatinine, a marker of acute kidney injury. An inverse correlation was found between CD26 expressing cells and chemokine, MCP-1. Although not statistically significant, inverse trends were found between the CD26 expressing cells and CRP and ferritin, markers of systemic inflammation in COVID-19. Strong positive associations were found between the number of CD26 expressing cells and the number of lymphocytes, including a subset of CD73 expressing cells. The analysis also revealed a positive trend between the number of CD73 expressing cells and hospital-free days. Sex-specific association has also been found with higher absolute numbers of CD26 cells in females.

Further analysis revealed a significantly lower number of CD26 expressing cells in patients with critical illness compared to control subjects (Figure 2B), in ICU versus non-ICU patients (Figure 2C), and non-survivors compared to those who survived (Figure 2D). The percentage of CD26-expressing cells within the total lymphocytes subpopulation has also been decreased in patients with a critical illness (Figure 2E), ICU patients (Figure 2F), and non-survivors (Figure 2G).

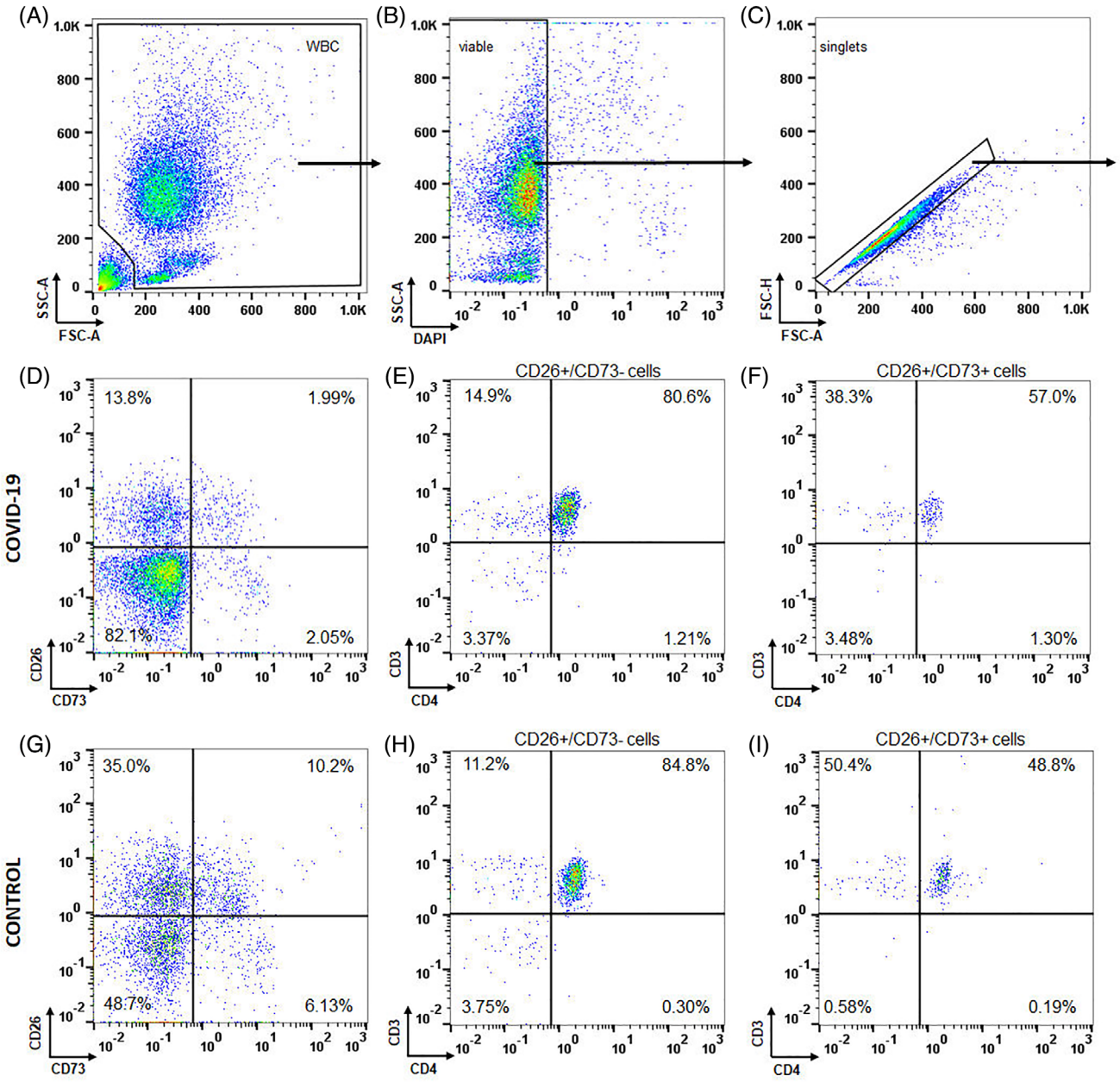

FIGURE 1 Lymphocyte subpopulation identification gating strategy. Representative flow cytometric plots. (A–C) Gating for single events, viable white blood cells (WBC). (D–I) Plots showing (D, G) subpopulations of $CD26^{pos}$ (upper left quadrant), $CD73^{pos}$ (lower right), and $CD26^{pos}CD73^{pos}$ (upper right) cells within WBC population, (E, H) subset of CD3/CD4 cells within $CD26^{pos}$ and (F, I) $CD26^{pos}CD73^{pos}$ cells in groups of (D, E, F) patients with COVID-19 and (G, H, I) control subjects [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | The number of CD26 cells increased during the first week in survivors

Next, we evaluated time-dependent changes in the number of CD26 expressing cells in patients with COVID-19. Subjects without data at multiple time points were excluded from the analysis; the majority of those subjects were patients with mild illness who were discharged before the collection of day 7 samples. We found an overall increase in the number of CD26 expressing cells within the first week (Figure 3A), but no significant increase in CD26 cells in the subgroup of non-survivors (Figure 3B), indicating a potentially important role of CD26 cells in disease resolution.

4 | DISCUSSION

Low number of circulating lymphocytes is associated with severe and critical COVID-19 illness [13–15]. While cytotoxic T cells are crucial for the prevention of virus replication [16], it has been shown that the activation of helper T cells contributes to the efficient immune

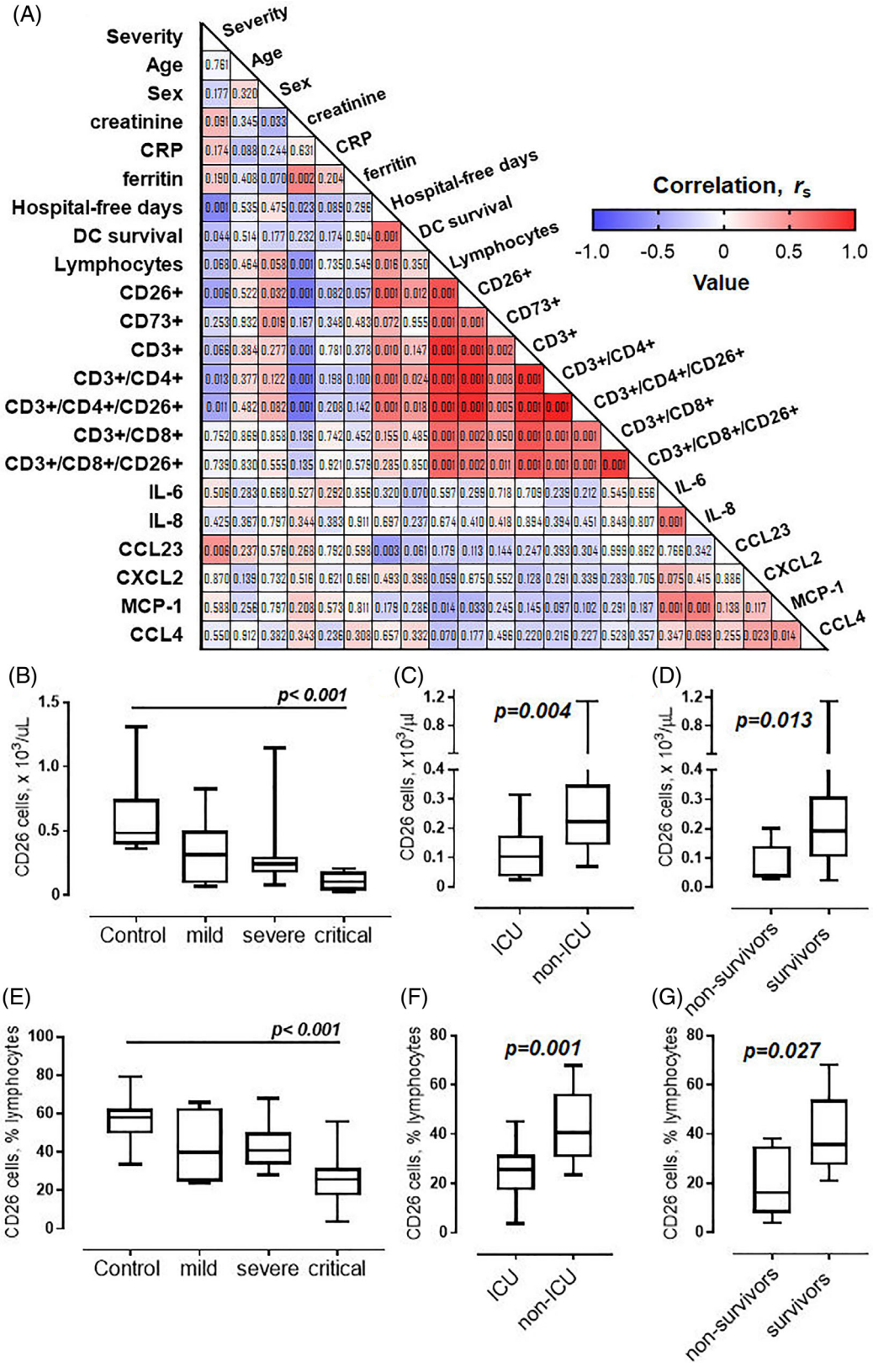

FIGURE 2 Legend on next page.

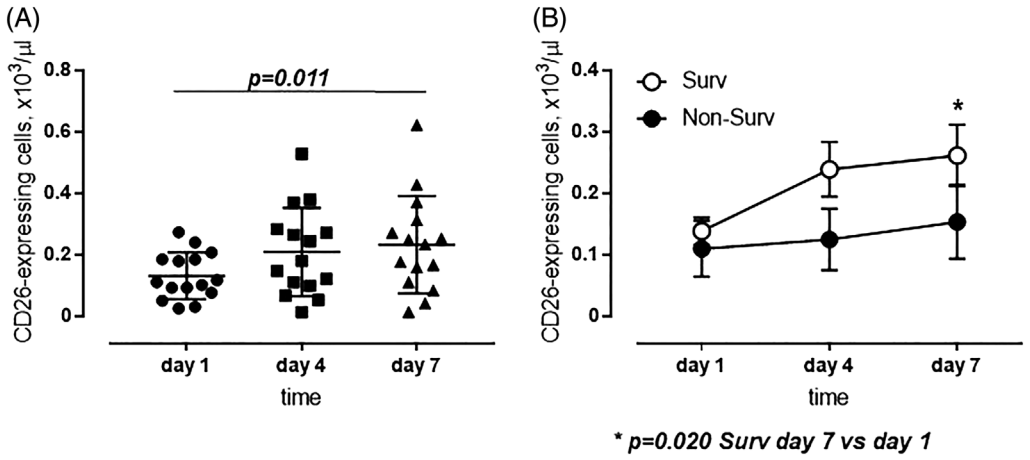

FIGURE 3 Survivors of COVID-19 infection show significant increases in CD26-expressing lymphocyte levels over time with standard treatment. (A) Number of CD26-expressing lymphocytes in patients with COVID-19 ($n = 15$; mild = 1, severe = 3, critical = 11) were determined on study days 1, 4, and 7. RM one-way ANOVA. (B) The number of CD26-expressing lymphocytes in survivors (surv, $n = 11$) and non-survivors (non-surv, $n = 4$) from study day 1 to day 7. Mean +/− SEM; RM two-way ANOVA, Tukey's multiple comparison test

response against SARS-Cov2 [17]. Our main finding is that the low number of CD4 T helper cells that express CD26 is associated with critical COVID-19 illness, and does not recover in non-survivors, suggesting that CD26-dependent signaling in T helpers may play an important role in disease resolution and recovery from acute SARS-CoV-2 infection.

Although the expression of CD26 can be found at low levels on natural killer cells, B cells, and monocytes [18–20], only T cells are characterized by high levels of CD26 cell surface expression [4]. T cells also contribute to the pool of circulating soluble CD26 [21]. CD26-mediated signaling is involved in the activation of both CD8 and CD4 T cells [5, 7, 12]. Our data demonstrate that CD4 T cells represent a major subset of CD26 expressing cells in the circulation of patients with COVID-19. We also found that the number of CD4 but not CD8 T cells expressing CD26 is inversely associated with poorer outcomes. While we did not assess T cell functional status, it is plausible to suggest that this relationship reflects the role of $CD26^{+}CD4^{+}$ T cells in the modulation of T cell cytotoxic activity, and that a higher number of these cells promote a more efficient immune response against SARS-Cov2.

The important role of CD26 expressing cells in the immune response to viral infections has been highlighted by previous studies. A unique pattern of CD26 expressing CD8 T cells has been identified in acutely resolved infection compared to chronic infections, indicating the potential role of CD26 expressing cells in infection prevention and resolution [22]. A recent study demonstrated a decreased number of CD26 expressing T cells in HIV, specifically in progressors, symptomatic individuals with high-viral loads [23]. However, in a different study, no differences were found between the control group and progressors [24]. Both studies performed characterization of CD26 cells in relatively small cohorts of subjects, which may partially explain the controversy. Our study adds to the increasing evidence that CD26 expressing cells play a role in the pathogenesis of viral infections, including SARS-Cov2, and requires further investigation.

Sex is a contributing factor in infectious disease pathology [25]. A higher number of CD26/CD4 T lymphocytes have been found in healthy women compared to men. However, this difference has been abrogated in progressive HIV-1 [26, 27]. In this context, it is interesting that several studies demonstrated lower HIV viral load in women early during infection and no differences in disease progression between the sexes [28]. Female sex is associated with lower risks of death and intensive care unit treatments in patients with COVID-19 [29]. In our study, we also found an association between sex and lymphocytes, with a higher number of CD26/CD4 T cells in women. Age represents another risk factor in COVID-19 [30]. A complex CD26 expression pattern was found on CD8 T lymphocytes associated with

FIGURE 2 Low levels of CD26-expressing lymphocytes are associated with the severity of COVID-19 disease and with poor outcome. (A) Heatmap correlation matrix of study day 1 lymphocytes and lymphocyte subpopulations, subject clinical parameters, and outcomes after hospitalization for COVID-19 infection. Color represents the spearman correlation coefficient. *p*-value is shown in each box. CRP, c-reactive protein; DC survival, survival to hospital discharge, categorical variable where 0-non-survivors and 1-survivors; lymphocytes, cells in "lymphocyte gate" defined as $SSC\text{-}a^{low}/CD14^{negative}$ cells, that include T and B lymphocytes and NK cells; sex, a categorical variable where 0-male and 1-female, positive correlation indicates higher number of CD26 cells in females. (B) Number of CD26-expressing lymphocytes in patients with COVID-19 by severity (study day 1; mild = 7, severe = 10, critical = 12) and control subjects ($n = 12$). (C–D) Number of CD26-expressing lymphocytes on study day 1 in (C) patients with COVID-19 admitted to the ICU ($n = 12$) and non-ICU patients ($n = 17$) and (D) patients who survived ($n = 24$) and non-survivors ($n = 5$) to discharge from the hospital. (E–G) Percentage of CD26-expressing cells within subpopulation of total lymphocytes. (B), (E) Kruskal-Wallis one-way ANOVA, Dunn's multiple comparison test. (C), (D), (F), (G). Mann Whitney test [Color figure can be viewed at wileyonlinelibrary.com]

a significant decrease in aging [31]. No differences, however, were found for CD26/CD4 cells. We also did not find any association between age and the number of CD26/CD4 T cells or the severity of COVID-19.

CD26 is also a binding protein for adenosine deaminase, an enzyme that catalyzes the conversion of adenosine to inosine [32]. Adenosine is an endogenous purine nucleoside characterized by potent immunosuppressive properties [33–36] and may mediate inhibition of effective cytotoxic T cells response against the SARS-Cov2. T cells are highly mobile; after egress from lymphoid organs, they circulate for a very short time before entering nonlymphoid tissue [37]. In agreement with the potential role of $CD4^{+}CD26^{+}$ T cells in the regulation of adenosine availability, we found that only a small fraction of these cells co-expressed CD73, an adenosine-generating enzyme. Our data also demonstrated that the total number of CD73 expressing cells is significantly lower compared to CD26 expressing immune cells in patients with COVID-19. No associations were found between the number of CD73 expressing cells and the severity of COVID-19 illness. However, our data demonstrated a trend toward a positive association between CD73 cells and hospital-free days, indicating the potential role of these cells in recovery. It should be noted that the coordinated action of CD73 and adenosine deaminase results in the generation of inosine, a nucleoside that contributes to T cell cytotoxic activity [38] and down-regulation of inflammatory cytokine production [39].

CD26 has been described as a candidate binding target for the SARS-Cov2 spike protein [3, 40]. This has led to the hypothesis that CD26 may play a role in facilitating SARS-Cov2 entry into the cells. Several studies have demonstrated the beneficial effects of CD26 inhibition in COVID-19 [41–43]. On the other side, it has been shown that the inhibition of CD26 is associated with increased coagulation and the development of venous thrombosis [2]. Our new data indicates the necessity of further investigations to determine the impact of CD26 inhibitors on CD4 T cell number and activation status before their clinical use in patients with COVID-19.

In conclusion, we demonstrate a strong inverse association between $CD26^{+}CD4^{+}$ T lymphocytes and severity of COVID-19 illness and mortality, indicating the important role of T helper cells in the resolution of and recovery from COVID-19 disease. The roles of myeloid cells, neutrophils and monocytes, and their secreted soluble factors (including IL-1β, IL-6, IL-8, and TNFα) have been well-studied in COVID-19 disease [44]. Many current therapies are focused on the inhibition of myeloid-driven inflammation [45–47]. Our data indicate that therapeutic strategies targeting the stimulation of T helpers, specifically CD26 expressing lymphocytes, might be beneficial in the development of effective treatments against COVID-19. Time-dependent recovery in the number of CD26 expressing cells may also be an important marker of recovery or treatment efficacy for individual patients. More research into the cellular mechanisms of COVID-19 infection and its successful resolution is warranted.

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AUTHOR CONTRIBUTIONS

Joanne T deKay: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Teresa L May:** Conceptualization (equal); data curation (equal); methodology (equal); resources (equal); writing – review and editing (equal). **Richard R Riker:** Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); resources (equal); writing – review and editing (equal). **Jonathan Rud:** Conceptualization (equal); data curation (equal); resources (equal); writing – review and editing (equal). **David J Gagnon:** Conceptualization (equal); data curation (equal); resources (equal); writing – review and editing (equal). **Douglas B Sawyer:** Conceptualization (equal); data curation (equal); methodology (equal); supervision (equal); writing – review and editing (equal). **David B Seder:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Sergey Ryzhov:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

CONFLICT OF INTEREST

The co-authors have no disclosures.

PEER REVIEW

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ORCID

Sergey Ryzhov https://orcid.org/0000-0002-7930-9814

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