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Cancer-driven changes link T cell frequency to muscle strength in people with cancer: a pilot study

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Abstract

Background Tumour growth can promote the loss of muscle mass and function. This is particularly disturbing because overall survival is significantly reduced in people with weaker and smaller skeletal muscle. The risk of cancer is also greater in people who are immune deficient. Muscle wasting in mice with cancer can be inhibited by infusion of CD4$^+$ precursor T cells that restore balanced ratios of naïve, memory, and regulatory T cells. These data are consistent with the hypothesis that stronger anti-cancer T cell immunity leads to improved muscle mass and function. As a first step to testing this hypothesis, we determined whether levels of circulating T cell subsets correlate with levels of muscle strength in people with cancer.
Methods The frequency of circulating CD4$^+$ and CD8$^+$ naïve, memory, and regulatory T cell subsets was quantified in 11 men with gastrointestinal cancer (aged 59.3 ± 10.1 years) and nine men without cancer (aged 60 ± 13 years), using flow cytometry. T cell marker expression was determined using real-time PCR and western blot analyses in whole blood and peripheral blood mononuclear cells. Handgrip strength, one-repetition maximum chest press, and knee extension tests were used to determine muscle strength. Performance was determined using a stair climb test. Body composition was determined using dual-energy X-ray absorptiometry scan. The Karnofsky and ECOG scales were used to assess functional impairment. Correlations between frequencies of cell subsets with strength, performance, and body composition were determined using regression analyses.
Results Our data show significant correlations between (i) higher frequencies of CD8$^+$ naïve ($P = 0.02$) and effector memory ($P = 0.003$) T cells and lower frequencies of CD8$^+$ central memory T cells ($P = 0.002$) with stronger handgrip strength, (ii) lower frequency of regulatory cells with greater lean mass index ($P = 0.04$), (iii) lower frequency of CD8$^+$ T cells that express CD95 with greater stair climb power ($P = 0.003$), (iv) higher frequency of T cells that co-express CD197 and CD45RA and greater one-repetition maximum knee extension strength ($P = 0.008$), and (iv) higher expression of CD4 in whole blood with greater functional impairment ($P = 0.004$) in people with cancer.
Conclusions We have identified significant correlations between levels of T cell populations and muscle strength, performance, and body composition in people with cancer. These data justify a follow-up study with a larger cohort to test the validity of the findings.

Keywords Cancer; Muscle; T cells; Flow cytometry; Correlations

Introduction

Over the past four decades, many studies have focused on identifying changes in the circulating T cell immune profile in people

with cancer, compared with healthy controls. These studies have reported an elevated frequency of several cell subsets including CD8$^+$ and CD4$^+$ T cells, Treg cells,[1] and pro-inflammatory Th17 cells[2] in people with cancer. Other studies have reported a

reduction in B cells[1] in people with cancer. To better define the role for particular T cell subsets in cancer progression, investigators have correlated the relative frequency of circulating T cell subsets with length of overall survival and disease-free survival. Using this approach, low levels of CD8^{+} naïve T cells, high levels of CD8^{+} memory cells,[3] and high levels of Treg subsets[4] have been found to correlate with poor prognosis. In similar studies, elevated levels of interleukin (IL) 6[5] and pro-inflammatory Th1-type cytokines[6,7] and low levels of Th2-type and regulatory type cytokines (IL-4, IL-5, IL-10, and TGF-β)[6] correlate with favourable outcome. Overall, the data show that the capacity to mount a pro-inflammatory T cell response in the absence of an anti-inflammatory Treg and Th2-type response bodes well for a more positive outcome.

In people with cancer, reduced muscle mass and strength are also negatively associated with disease-free survival and overall survival.[8–12] Tumour growth is known to promote loss of muscle mass and function; however, the specific mechanisms mediating this effect are incompletely understood.[13] Cancer-specific T cell responses that target cancer cells might indirectly protect muscle mass and function by reducing tumour burden.[14] During the later stages of the disease, a non-specific increase in overall immune activity might have the opposite effect on muscle function by increasing the metabolic burden on the body, resulting in muscle loss.[14] Thus, the immune response can be beneficial to muscle function if directed towards the cancer and detrimental if non-specifically elevated. Currently, there is no way to distinguish T cell profiles that are potentially beneficial from those that are potentially destructive for muscle function.

There is no 'gold standard' measure of muscle strength and function in people with cancer. However, common tests used in the setting of chronic disease include handgrip strength (HGS), chest press, knee extension, and stair climb power (SCP) tests. The HGS shows a strong correlation with all-cause mortality,[15,16] and resistance training in people with cancer increases upper and lower body muscular strength and improves quality of life.[17–19] The SCP is a measure of lower extremity balance, strength, and power. Elevated stair climb time correlates with elevated heart rate and slower recovery in people recovering from surgery.[20–22] Also, HGS and stair climb time (SCT) have been used as primary outcome measures in phases II–III clinical trials for anabolic agents in development for the treatment of cancer-induced cachexia.[23–27]

Previously published work from our group has shown that injecting CD4^{+} precursor T cells into mice with cancer can both re-establish balanced numbers of naïve, memory, and regulatory T cells[28,29] and protect from muscle wasting.[30] To our knowledge, these data were the first to show a link between muscle health and T cell homeostasis in mice. In the current study, we test whether there is an association between immune deficiency and muscle function by measuring correlations between levels of circulating T cell subsets and levels of muscle strength and performance. These data will allow us to distinguish T cell profiles that are potentially beneficial from those that are potentially destructive for muscle health. Specifically, we predict that strong anti-cancer T cell profiles will be associated with higher levels of muscle strength.

Materials and methods

Two study groups, one with and one without cancer

Age and sex differences can significantly contribute to muscle wasting.[31] Therefore, in this study, we matched the two groups for sex (all subjects were male) and age. Analysis after unblinding the study showed that the two groups were also matched for body mass index (BMI) and body composition (*Table* 1). Whole blood was collected in heparin from four people with cancer and six age-matched men without cancer at the Michael E. DeBakey VA Medical Center in Houston, TX, and from seven people with cancer and four age-matched men without cancer at the VA Puget Sound Health Care System in Seattle, WA.

Inclusion criteria for the cancer group

Cancer diagnosis was confirmed using a combination of histological, cytological, and image based-methods. All subjects were willing and able to provide informed consent. Cancer type and stage, and time between diagnosis and blood collection are shown in *Table* 2. For the non-cancer group, no history of active cancer (other than non-melanoma skin cancer) and willing and able to provide informed consent.

Exclusion criteria for both groups

Non-cancer causes of muscle mass loss including liver disease (aspartate aminotransferase or alanine aminotransferase, equal to or more than 3 times normal levels), renal failure (creatinine equal to or more than 2.5 mg/dL), untreated thyroid disease, classes III–IV congestive heart failure, AIDS, severe chronic obstructive pulmonary disease requiring use of home O_2, active and uncontrolled infection, uncontrolled diabetes mellitus, current use (within 1 month) of testosterone and high-dose steroids (20 mg of prednisone per day for more than 1 month), or megestrol. People with cancer were also excluded if they had been diagnosed with any other cancer within the past 5 years, except non-melanoma skin cancer.

Blood and clinical data collection

Fresh blood collected from all study participants with cancer and those without cancer was shipped overnight to San Diego

Table 1. Muscle mass, strength, and performance measurements in people with cancer-matched and non-cancer-matched controls

	Non-cancer[a]	Cancer patients[b]	N (non-cancer/cancer)[c]	P-value[d]
Age (years)[e]	60 ± 13.03[f]	59.273 ± 10.09	(9)/(11)	0.887
BMI (kg/m^2)	27.48 ± 5.75	25.24 ± 3.52	(9)/(11)	0.291
Body composition				
Lean mass index (kg/m^2)[h]	19.27 ± 2.9	17.97 ± 2.68	(9)/(11)	0.303
Fat mass index (kg/m^2)[h]	7.35 ± 3.6	6.45 ± 1.9	(9)/(11)	0.474
Bone mineral density (kg/m^2)[i]	1.19 ± 0.15	1.16 ± 0.08	(9)/(11)	0.597
Muscle strength and performance				
Handgrip strength (non-dominant) (kg)	41.45 ± 9.84	39.22 ± 9.64	(9)/(9)	0.625
Handgrip strength (dominant) (kg)	45.9 ± 10.75	36.35 ± 8.21	(9)/(10)	0.039
Chest press (kg)	58.9 ± 31.02	34.78 ± 17.54	(9)/(09)	0.056
Knee extension (kg)	70.7 ± 32.28	48.9 ± 22.31	(9)/(10)	0.096
Stair climb power (W)	476.70 ± 167.65	323.32 ± 164.02	(9)/(10)	0.053

BMI, body mass index.
[a]Nine subjects without cancer matched with the patient group for age, gender, and BMI.
[b]Eleven patients diagnosed with cancer.
[c]Number of subjects in each group.
[d]Statistical significance between groups calculated using the unpaired Student's *t*-test.
[e]Age range is 40–70 years.
[f]Mean ± standard deviation.
[g]Calculated using the dual-energy X-ray absorptiometry scan to determine total lean mass divided by the height in square metres.
[h]Calculated using the dual-energy X-ray absorptiometry scan to determine total fat mass divided by the height in square metres.
[i]Calculated using the dual-energy X-ray absorptiometry scan to determine bone mineral density divided by the height in square metres.

Biomedical Research Institute at ambient temperature and processed on arrival. Peripheral blood mononuclear cells (PBMCs) were isolated using ficoll and either used immediately or frozen in liquid nitrogen, as indicated for each experiment. Freeze thaw changes the frequency of cells that express CD62L and CD197. Therefore, these cell subsets were quantified only in fresh blood. The study subjects' age, BMI, lean mass index (LMI), fat mass index (FMI), bone mineral density (BMD), and various measures of muscle strength were recorded on the day of blood draw. The study was blinded. Human Subjects protocols and consent forms were reviewed and approved by each institution's IRBs and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Peripheral blood mononuclear cell used as positive controls for flow cytometry staining and cell function

Frozen stocks of well-characterized PBMC from healthy subjects aged 19–30 years were used as positive controls for monoclonal antibody staining in each experiment. Whole blood from these donors was obtained from the Normal Blood Donor Program at The Scripps Research Institute. Human Subjects protocols and consent forms were reviewed and approved by both The Scripps Research Institute IRB and San Diego Biomedical Research Institute IRB and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Whole blood was collected in heparin and processed

Table 2. Description of cancer type and stage in the patients studied

Primary site[a]	Stage[b]	Metastasis[c]	Metastasis site[d]	Time (days) between diagnosis and study[e]
Small bowel	IV	Yes	Bone, LN	246
Pancreas	IIB	No	NA	207
Pancreas		No	NA	104
Gastric	IV	Yes	Diaphragm, pelvis, anterior abdominal wall	29
Pancreas	IV	Yes	Liver, lung	236
Esophageal	IV	Yes	LN	361
Rectal	IV	Yes	Abdomen	531
Rectal	IIIB	No	NA	1093
Gastric	IV	Yes	Liver, abdomen, lung indeterminate	85
NSCLC	IIB	No	NA	48
Nasopharyngeal	IVB	No	NA	958

LN, lymph nodes; NA, not applicable—no metastasis; NSCLC, non-small cell lung cancer.
[a]The site where the first cancer was detected.
[b]Cancer stage when blood sample was collected.
[c]Presence or absence of metastasis.
[d]The site where metastasis was detected.
[e]Time in days between first diagnosis with cancer and first study visit.

within 2 h. PBMCs were isolated using standard methods and frozen in liquid nitrogen.

Reagents used to identify cell subsets in fresh and frozen peripheral blood mononuclear cells

Fluorochromes, vendors, catalogue numbers, and registry identifiers for all antibodies used in flow cytometry experiments, including isotype control antibodies, are as shown in Supporting Information, *Table S1*. Of the more than 40 cell subsets quantified, data are shown for only those that show significant correlations with one or more measures of muscle function.

Identification of CD4+ and CD8+ naïve and memory T cell subsets and CD4+ regulatory T cell subsets

Using PBMC isolated from fresh blood, the various naïve and memory (*Table* 3A) and regulatory (*Table* 3B) T cell subsets were identified using standard published makers as follows[32–38]: Cells were labelled for CD3 and either CD4 or CD8 with combinations of CD45RA CD45RO CD197

CD28 CD31 CD95 CD62L and Ki67 to identify total naïve ($CD45RA^+$ $CD197^+$ $CD28^+$), total memory ($CD45RA^-$ $CD45RO^+$), central memory (CM, $CD45RA^-$ $CD197^+$ $CD28^+$), effector memory (EM, $CD45RA^-$ $CD197^-$ $CD28^{+/-}$), $CD45RA^+$ effector memory (EMRA, $CD45RA^+$ $CD197^-$, $CD28^-$), and stem cell-like memory (T_{SCM}, $CD45RA^+$ $CD45RO^-$ $CD197^+$ $CD95^+$) cells. Recent thymic emigrants (RTEs) are a subset of naïve cells and are identified as $CD45RA^+$ $CD45RO^-$ CD197 and $CD31^+$.[39,40] Ki67 expression was used to determine whether RTEs are dividing ($Ki67^+$) or not dividing ($Ki67^-$). CD62L expression was also used to distinguish activated ($CD62L^-$) from resting ($CD62L^+$) cells. Regulatory cells (Treg) were identified by their high expression of CD25 and low expression of CD127 as described previously using the BD Biosciences Treg cocktail that includes antibodies specific for CD4, CD127, and CD25 with CD3. $CD25^+$ $CD127^{hi}$ cells were also identified using the BD Biosciences Treg cocktail as $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{hi}$.[41,42] Activated Treg were identified as $CD3^+$ $CD4^+$ $CD45RA^-$ $Foxp3^{hi}$.[34] Data were acquired on an LSRFortessa and analysed using FlowJo version 10 (Ashland, OR). Isotype controls were used in every experiment and for every antigen-specific antibody. All naïve, memory, and regulatory T cell subsets were analysed in blinded fashion.

Table 3. T cell populations quantified in the blinded study

Cell subset names[a]	Antibody specificities used[b]
A. CD4 and CD8 naïve and memory T cell subsets	
CD8 subsets	
Total naive	$CD3^+$ $CD8^+$ $CD45RA^+$ $CD197^+$ $CD28^+$
Recent thymic emigrants	$CD3^+$ $CD8^+$ $CD45RA^+$ $CD197^+$ $CD45RO^-$ $CD31^+$ $Ki67^-$
Total memory	$CD3^+$ $CD8^+$ $CD45RA^-$ $CD45RO^+$
Central memory	$CD3^+$ $CD8^+$ $CD45RA^-$ $CD197^+$ $CD28^+$
Effector memory (EM)	$CD3^+$ $CD8^+$ $CD45RA^-$ $CD197^-$ $CD28^{+/-}$
$CD45RA^+$ EM (EMRA)	$CD3^+$ $CD8^+$ $CD45RA^+$ $CD197^-$ $CD28^-$
Stem cell-like memory (T_{SCM})	$CD3^+$ $CD8^+$ $CD45RA^+$ $CD45RO^-$ $CD197^+$ $CD95^+$
CD4 subsets	
Total naive	$CD3^+$ $CD4^+$ $CD45RA^+$ $CD197^+$ $CD28^+$
Recent thymic emigrants	$CD3^+$ $CD4^+$ $CD45RA^+$ $CD197^+$ $CD45RO^-$ $CD31^+$ $Ki67^-$
Total memory	$CD3^+$ $CD4^+$ $CD45RA^-$ $CD45RO^+$
Central memory	$CD3^+$ $CD4^+$ $CD45RA^-$ $CD197^+$ $CD28^+$
Effector memory (EM)	$CD3^+$ $CD4^+$ $CD45RA^-$ $CD197^-$ $CD28^{+/-}$
$CD45RA^+$ EM (EMRA)	$CD3^+$ $CD4^+$ $CD45RA^+$ $CD197^-$ $CD28^-$
Stem cell-like memory (T_{SCM})	$CD3^+$ $CD4^+$ $CD45RA^+$ $CD45RO^-$ $CD197^+$ $CD95^+$
B. CD4 regulatory T cell subsets	
Treg	$CD3^+$ $CD4^+$ $CD25^{hi}$ $CD127^{low}$
Activated Treg	$CD3^+$ $CD4^+$ $CD45RA^-$ $Foxp3^{hi}$
$CD25^+CD127^{hi}$	$CD3^+$ $CD4^+$ $CD25^+$ $CD127^{hi}$
C. Tc1/Th1, Tc2/Th2 and Tc17/Th17 cells and cytokines[c]	
CD8 subsets	
Tc1 cells	$CD3^+$ $CD8^+$ $IFN\text{-}\gamma^+$ $T\text{-}bet^+$
Tc2 cells	$CD3^+$ $CD8^+$ $IL\text{-}4^+$ $GATA\text{-}3^+$
Tc17 cells	$CD3^+$ $CD8^+$ $IL\text{-}17^+$ $ROR\gamma t^+$
CD4 subsets	
Tc1 cells	$CD3^+$ $CD4^+$ $IFN\text{-}\gamma^+$ $T\text{-}bet^+$
Tc2 cells	$CD3^+$ $CD4^+$ $IL\text{-}4^+$ $GATA\text{-}3^+$
Tc17 cells	$CD3^+$ $CD4^+$ $IL\text{-}17^+$ $ROR\gamma t^+$

[a]Well-established and published $CD8^+$ and $CD4^+$ T cell subsets.
[b]Published and validated markers to identify $CD4^+$ and $CD8^+$ T cell subsets.
[c]Pro-inflammatory IFN-γ, TNF-α, IL-17A, IL-2, and anti-inflammatory IL-4.

Identification of CD95 expressing non-T cells

CD95 expressing non-T cells were identified in PBMC isolated from fresh blood as CD3$^-$ CD95$^+$ cells and their relative frequency within the total PBMC population determined by flow cytometry as described for the T cell subsets.

Identification of Th1/Tc1, Th2/Tc2, and Th17/Tc17 CD4$^+$ T cell subsets

Frozen PBMCs were thawed, washed twice in RPMI (Invitrogen) with 10% human AB serum, and rested at 37°C overnight. Cells were suspended in RPMI with 10% human AB serum, HEPES (Gibco BRL), glutamine, penicillin, streptomycin (Irvine Scientific), and 2-mercaptoethanol (Sigma-Aldrich) and cultured in 24-well plates at a concentration of $1–3 \times 10^6$ cells/mL with 50 ng/mL PMA (Sigma) and 1 mM ionomycin (Sigma). One microlitre of Brefeldin A (BD Bioscience) per millitre medium was added at the beginning of the culture. After 4 h cultured cells were washed twice. Th1/Tc1, Th2/Tc2, and Th17/Tc17 cells were identified by their intracellular co-expression of IFN-γ and the transcription factor T-bet for Th1/Tc1,[36] IL-4 and GATA-3 for Th2/Tc2,[43] and IL-17 and the transcription factor RORγt for Th17/Tc17[44] (*Table* 3C). The frequency of cells co-expressing cytokines and transcription factors was measured by intracellular stain and flow cytometry. In addition, the relative frequency of CD4$^+$ or CD8$^+$ T cells that express IL-2, IL-4, IFN-γ, TNF-α, or IL-17 was determined by intracellular stain. All cytokine-positive T cell subsets were analysed in blinded fashion.

RNA extraction from whole blood

Five hundred microlitres of whole blood was mixed thoroughly in 1300 μL of RNALater (Ambion, Cat #AM7021). The samples were stored at −20°C until extraction. RNA was extracted from the blood using the RiboPure Blood Kit following the manufacturer's instructions (Ambion Cat # AM1928). The RNA concentration was determined using the Thermo Scientific NanoDrop Lite Spectrophotometer.

cDNA extraction and qPCR

One hundred fifty nanograms of RNA was used to convert to cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit following the manufacturer's instructions (Cat # 4368814). The cDNA thus obtained was diluted at 1:10, 1:100, and 1:1000. The housekeeping gene, 18 s, was run at all three dilutions to ensure the quality of the cDNA. Five microlitres of the 1:100 cDNA reaction was used to perform qPCR on the gene of interest. For each qPCR reaction, the 5 μL cDNA was added to 10 μL of iTaq Universal SYBR Green Supermix (2X) (Biorad Cat #1725120), 1.5 μL of each primer (375 nM concentration), and 2 μL of nuclease free water. Thus, a total volume of 20 μL of qPCR mix was run on the BioRad CFX connect qPCR machine. The qPCR data for each gene were analysed using the double delta Ct method.

qPCR primer design and sequences

Primers were designed by downloading the gene sequence of the gene of interest from the CCDS database. Each sequence was entered into Primer 3 software, and the suggested primer pairs were blasted through the NCBI (BLAST) and UCSC genome browser (BLAT) database. The primer sequences (Invitrogen) were diluted to a stock solution of 100 μM in TE buffer. A working solution of 5 μM was used for the qPCR reaction. Primer sequences used are listed as follows: CD197 Fwd: 5'GATGCGATGCTCTCTCATCA 3'; CD197 Rev: 5'TGTAGGGCAGCTGGAAGACT 3'; CD4 Fwd: 5'AGGAAGT GAACCTGGTGGTG 3'; CD4 Rev: 5'CTCAGCAGACACTGCCACAT 3'; CD95 Fwd: 5'CAAGGGATTGGAATTGAGGA 3'; and CD95 Rev: 5'TGGAAGAAAAATGGGCTTTG 3'.

Protein extraction and western blot from peripheral blood monocytes (peripheral blood mononuclear cell)

Protein was extracted from frozen PBMC cells using the TriReagent (Cat #AM9738) extraction method using the TriReagent DNA/Protein Isolation Protocol. The protein extracted was dissolved in 20–50 μL of 8 M Urea/20 mM DTT solution and the concentration of protein determined using the Bradford protein assay. The samples were stored at −80°C. For western blot, the samples were thawed, and 30 μg of protein was mixed with 4× loading dye and loaded on to a gradient 4–20% Tris Glycine Acrylamide gel (Invitrogen Cat #XP04205BOX) and run at 180 V for ~1.5 h. The gels were transferred onto a PVDF membrane at 80 mA overnight. The membrane was stained with Ponceau stain to ensure that no air bubbles had interfered with the transfer. The membrane was then blocked in 5% milk solution for 1 h followed by the antibodies of interest—actin (1:2000 for 1 h); anti-CD197 (1:10 000 for 1 h); anti-CD95 (1:1000 for 1 h); anti-caspase 3 (1:1000 for 1 h); and anti-CD45RA (1:750 for 1 h). Corresponding secondary antibodies for rabbit (1:5000 dilution) and mouse (1:2000 dilution) were used for 30 min for actin and 1 h for all other antibodies. Refer to Supporting Information, *Table S1* for a detailed description of the antibodies used. The membrane was visualized using a SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific Cat #34577) and CL-Xposure film (Thermo Scientific Cat #34090). Protein quantification was achieved

using the ImageJ software (NIH, Bethesda). The non-cancer and cancer samples were run on the same gel with samples from three 19- to 29-year-old healthy donors as positive controls for detection of each protein. Protein was normalized by dividing the mean densitometry of each experimental protein by the mean densitometry of actin within each sample and the data compared between the cancer and the non-cancer groups using GraphPad Prism v6 for Mac.

Measurement of lean and fat mass indices

Lean mass index, FMI, and BMD were determined by performing a dual-energy X-ray absorptiometry scan on each subject. This method uses low-level radiation to determine bone mineral content (kilograms), fat mass (kilograms), and lean mass (kilograms). Indices for lean and fat mass were calculated using the formula: (Lean or Fat Mass)/(Height)^2. Units for LMI, FMI, and BMD are kg/m^2.[45] Scans were performed after an overnight fast and before any physical performance tests on the morning of the study visit; each subject wore hospital pants and gown during the scan.[46]

Handgrip, chest press, and knee extension strength measurements

HGS was measured using a hand-held dynamometer (Jamar Hydraulic Dynamometer, J.A. Preston Corp., Clifton, NJ) as we have previously described.[23] The subjects were seated with the elbows at 90° and the wrist in a neutral position. Three trials for HGS were conducted to obtain a stable and accurate reading. HGS was performed on both the dominant and non-dominant hands. Bilateral one-repetition maximum (1-RM) was determined in an upright seated position for *Chest Press and Knee Extension* exercises using pneumatic strength training equipment (Keiser Sports Health Equipment, Inc., Fresno, CA). For each exercise, subjects performed one warm-up set of 7–10 repetitions at ~50% perceived maximal effort, followed by 1 min of rest, and then a second warm-up set of five repetitions at ~70% perceived maximal effort. Single repetitions were then performed, with 30 s to 1 min of rest in between, until maximal effort was achieved while maintaining proper range of motion 1-RM (i.e. for Knee Extension, knee angle started from ~45° and ended at ~170°). The 1-RM was measured in kilograms.

Performance test—stair climb power

Stair climb power test allows measuring the maximal anaerobic power of the involved muscles. Two to three practice trials were allowed so that the subjects gain a good control of performing the technique. Subjects ascend a set of stairs at the highest possible speed, according to their capabilities. The stairs consist of 13 steps, 15.3 cm each, thus covering a total vertical distance of 1.99 m. Anaerobic power in Watts (W) is calculated by the following formula: (body mass × 9.81 × vertical distance)/time where body mass, vertical distance (i.e. 1.99 m), and time to completion are expressed in kilograms, metres, and seconds, respectively, and 9.81 m/s^2 represents the acceleration of gravity.

Performance status measures

Karnofsky Performance Status Scale is a standard scale used to quantify functional impairment in patients with cancer.[47] There are 10 categories of increasing impairment in increments of 10 where a score of 100 indicates no impairment and a score of 0 is death. Each study subject was asked to place themselves into one of the categories and the score recorded. *ECOG Performance Status* was used to assess patient functional capacity.[48] Patients are given a grade from 0 (fully active) to 5 (dead) based on their ability to perform daily activities. Both tests have been well validated and are used routinely in clinical settings and in clinical trials.[49,50]

Statistics

Multiple groups were analysed using one-way analysis of variance or two-way analysis of variance. Comparisons between groups were analysed using Tukey post hoc. A *P* value of less than 0.05 is considered statistically significant. The level of statistical significance is indicated on the figures as $^*P = 0.05–0.01$, $^{**}P = 0.009–0.001$, $^{***}P = 0.0009–0.0001$, and $^{****}P < 0.0001$. Associations of muscle strength and mass with cell subset relative frequency were assessed using Spearman correlations (*r*). The Spearman correlation was used because it is a non-parametric correlation test that can be used when the assumptions of homogeneity of variance and normal distribution of errors are not met, or when the relationship is monotonic, but non-linear. In this paper, most relationships were non-linear, and some datasets demonstrated heterogeneity of variance. Regression lines shown on the correlation figures are illustrative only because most relationships were non-linear. These analyses were performed with GraphPad Prism and IBM Corporation, 2012.

Results

Muscle strength, performance, and body composition measurements in cancer and non-cancer participants

During recruitment, the two study groups were matched for age and sex. Subjects were men between 40 and 70 years

of age. Muscle strength, performance, BMI, and body composition were compared between groups after the study was unblinded. Body composition parameters including LMI, FMI, and BMD were similar between groups (*Table* 1). Non-dominant and dominant HGS, chest press, and knee extension were used to assess muscle strength, and SCP to assess performance. Of these measures, the dominant HGS was significantly different (lower) in the group with cancer compared with non-cancer controls. Chest press, knee extension, and SCP were also lower but did not reach significance. The non-dominant HGS was not different between groups (*Table* 1). The finding that muscle strength measured using HGS is significantly different between groups ($P = 0.039$), but other measures of muscle strength, performance, and mass are not different suggesting that, overall, the group with cancer is not cachexic but rather in the early stages of muscle impairment.

CD4$^+$ and CD8$^+$ naïve and memory T cell subsets

Of the naïve and memory T cell subsets measured (*Table* 2A), CD8$^+$ RTE, CM, and EM frequency correlates significantly with HGS, and RTE with 1-RM chest press strength in participants with cancer, but not in matched controls; in cancer participants, stronger HGS correlates with more CD8$^+$ RTE, fewer CD8$^+$ CM, and more CD8$^+$ EM (*Figure* 1A), but not with CD4$^+$ RTE, CM, and EM (data not shown). Greater 1-RM chest press strength also correlates with more CD8$^+$ RTE (*Figure* 1A), but not with CD8$^+$ CM and EM (*Figure* 1A), or CD4$^+$ cell

Figure 1 Handgrip strength (HGS) and one-repetition maximum chest press strength correlate with the frequency of circulating recent thymic emigrants (RTEs), central memory (CM), and effector memory (EM) in subjects with, but not without, cancer. Peripheral blood mononuclear cells were isolated from fresh whole blood and labelled for naïve and memory cell subsets as described in section and *Table* 2. Panel A shows the correlation between the frequency of naïve CD8$^+$ T cells that express CD31 but not Ki67 (CD8$^+$ RTE, *n* = 7), CD8$^+$ T cells that are CM (CD8$^+$ CM, *n* = 9), and CD8$^+$ memory cells that are EM (CD8$^+$ EM, *n* = 9) and either HGS or chest press strength in the cancer group. Each symbol represents a patient. Spearman correlation (*r*) and statistical significance (*P*) are shown on each panel in red for the cancer group and in black for the non-cancer group. (B) The frequency of each cell subset in people with cancer is compared with non-cancer-matched controls using the Student's *t*-test. Data shown are mean ± SEM. There is no statistically significant difference between groups.
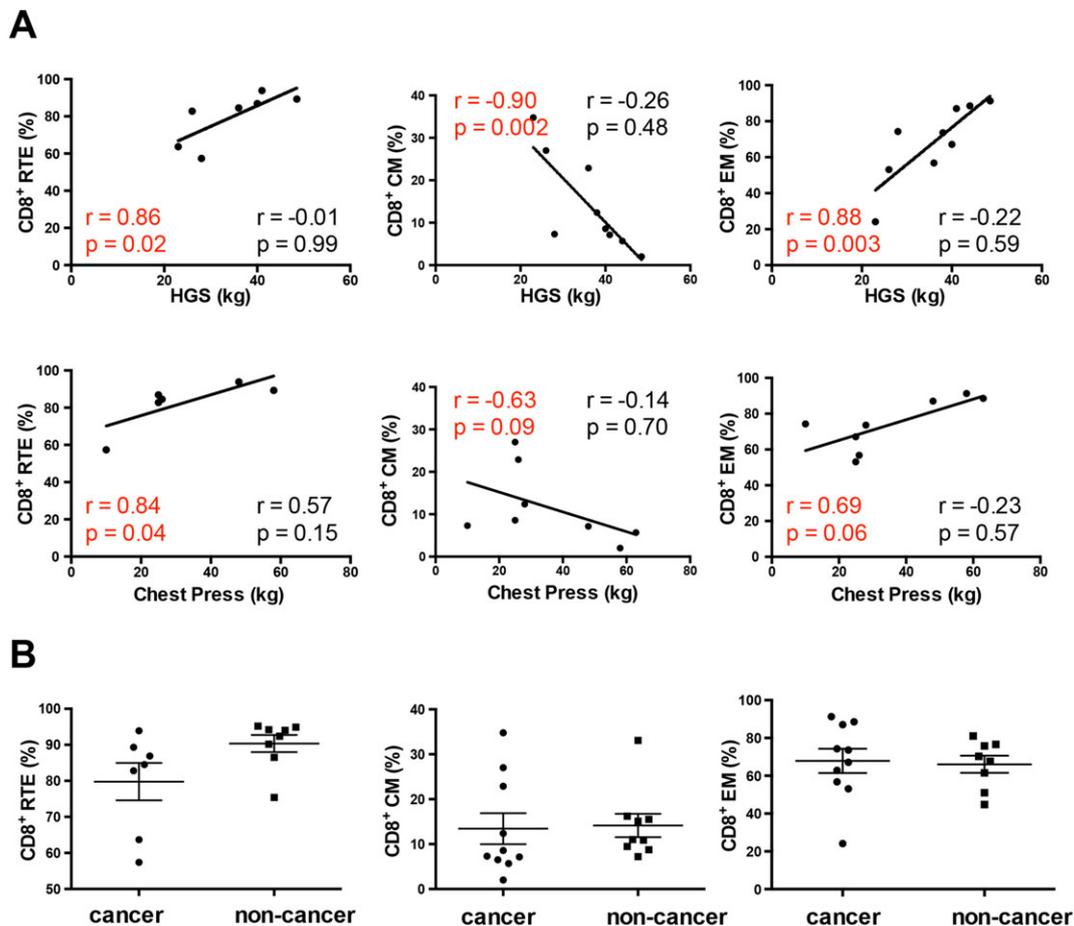
subsets (data not shown). 1-RM knee extension strength and SCP do not correlate with any of these cell subsets (data not shown). The gates used to identify RTE, CM, and EM, and representative examples of each cell subset, are shown in Supporting Information, *Figure S1*.

There was no difference in the frequency of cell subsets between the groups with and without cancer (*Figure* 1B), neither were any significant correlations found between these cell subsets and any muscle measures in the non-cancer matched control group (*P* and *r* values for non-cancer control group shown in black on each panel in *Figure* 1A). These data indicate that the correlations between RTE, CM, and EM cell subset frequency and muscle strength were related to upper body strength (HGS and chest press) and dependent on the presence of cancer. Total naïve, total memory, EMRA, and T_{SCM} cell frequencies did not correlate with any measures of muscle strength, performance, or body composition in either the group with cancer or without cancer (data not shown). Numbers of total T cells ($772.2 \times 10^3 \pm 122.4 \times 10^3$ and $569.6 \times 10^3 \pm 257.0 \times 10^3$ per mL whole blood), CD4$^+$ T cells ($558.9 \times 10^3 \pm 99.9 \times 10^3$ and $419 \times 10^3 \pm 234.6 \times 10^3$ per mL whole blood), and CD8$^+$ T cells ($214.6 \times 10^3 \pm 36.3 \times 10^3$ and $168.1 \times 10^3 \pm 60.2 \times 10^3$ per mL blood) were also not significantly different between non-cancer and cancer groups, respectively.

Naïve and memory cell markers

The flow cytometry data were reanalysed to test the minimal number of markers necessary to identify cell populations that correlate with muscle strength. Because many of the markers used to identify naïve and memory T cell subsets are also expressed by non-T cells, we also tested correlations with non-T cells in this phase of our analysis. Of all of the marker combinations tested, five T cell and non-T cell populations were identified that correlate with muscle strength— CD45RA$^+$ CD197$^+$ T cells (CD3$^+$), non-T cells (CD3$^−$), CD8$^+$ CD95$^+$ cells, CD4$^+$ CD95$^+$ cells, and CD95$^+$ PBMC.

CD197 and CD45RA

Using only CD3, CD197, and CD45RA to identify cell populations, we found that the frequency of CD197$^+$ T cells that co-express CD45RA correlates significantly with 1-RM knee extension strength but not with SCP, HGS, and 1-RM chest press strength (*Figure* 2A). These data might suggest that the T cells subsets (RTE and CM) identified using the additional markers, CD31 and CD45RO for RTE, and CD28 for CM, shown in *Figure* 1, are important to the relationship between T cells and HGS and chest press capacity in people with cancer. Further analysis showed highly significant correlations between CD197$^+$ CD45RA$^+$ non-T cells and HGS, chest press strength, and SCP in people with cancer (*Figure* 2B). The correlation with knee extension is not significant

(*Figure* 2B). Correlations between cell populations and muscle strength do not exist if cell populations are identified using either CD197 or CD45RA alone. In addition, no correlations exist in the non-cancer control group (data not shown). Overall, the data are consistent with an effect of cancer on CD197 and CD45RA-expressing circulating T cells and non-T cells by a mechanism that is linked to muscle strength.

To assess whether the presence of cancer modulated the overall expression of either CD197 or CD45RA, mRNA levels of CD197 in whole blood, and protein levels of CD197 and CD45RA in PBMC, were determined in subjects with cancer-matched and non-cancer-matched controls and compared between groups. The expression of CD197 mRNA was significantly lower in the cancer group than in the non-cancer control group (*Figure* 3A). However, the CD197 protein levels were not different between groups, neither were CD45RA levels (*Figure* 3B). Of note, the protein levels of CD197 and CD45RA were highly variable within both groups (*Figure* 3C).

CD95

No significant correlations were found between T_{SCM} and any of the muscle function and body composition measures in our study (data not shown). However, the relative frequency of CD95$^+$ expressing CD8$^+$ T cells correlates strongly with SCP (*Figure* 4A), but not with with 1-RM knee extension strength (*Figure* 4B), upper body, HGS, and chest press (data not shown). Moreover, the correlations with SCP and 1-RM knee extension strength are only seen in the group with cancer. No significant correlations were seen with CD4$^+$ CD95$^+$ cells (data not shown). Overall, these data are consistent with the previously published finding that poor prognosis in people with cancer is associated with a significant increase in circulating CD95-expressing T cells.[51] Supporting Information, *Figure S2* shows the gating strategy used to identify CD8$^+$ and CD4$^+$ CD95-expressing T cells, and representative examples of each cell subset. Our further analyses show that the frequency of CD95$^+$ CD3$^−$ (non-T cells) cells also correlates with SCP (*Figure* 4C), but not with knee extension (*Figure* 4D), and again, only in the group with cancer.

The level of CD95 mRNA (*Figure* 5A) in whole blood and protein in PBMC (*Figure* 5B and 5C) was higher in the group with cancer compared with the non-cancer control group, but the difference was not significant. However, analyses showed strong correlations between levels of CD95 mRNA and 1-RM knee extension strength (*Figure* 5D) and SCP (*Figure* 5E). Our data also show a significant decrease in the level of full length caspase 3 in the group with cancer compared with without cancer (*Figure* 5B), suggesting that the link between higher frequency of CD95-expressing cells and muscle strength might involve a role for caspase 3.

Figure 2 T cells and non-T cells, identified by their co-expression of CD197 and CD45RA, correlate with muscle strength and performance. The frequency of CD197$^+$ CD3$^+$ (A) and CD197$^+$ CD3$^-$ (B) cells that express CD45RA. Correlations with handgrip strength (HGS, chest press strength, knee extension strength, and stair climb power (SCP) were calculated. Each symbol represents a participant. Spearman correlation (*r*) and statistical significance (*P*) are shown on each panel for the cancer group.
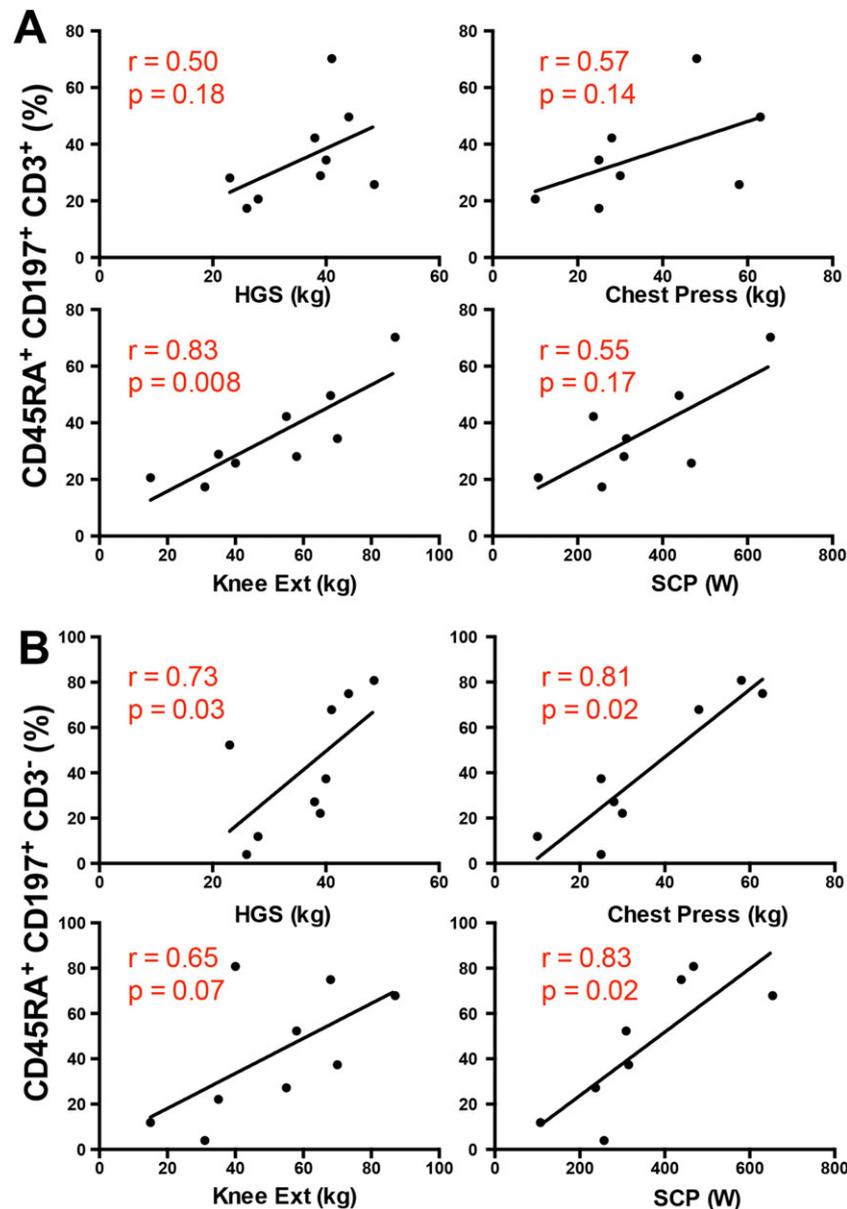

CD4$^+$ regulatory T cells

Treg frequency (*Figure* 6A) correlates significantly with LMI, but not with any muscle strength measures or performance (data not shown). The relative frequency of Treg was not different in the group with cancer compared with the non-cancer control group (*Figure* 6B). No additional correlations were found with other regulatory cell subsets or using fewer regulatory cell markers to identify cell populations. Schematics to show the gating strategy used to identify Treg cells,

and a representative example of the cell subset, is shown in Supporting Information, *Figure S3*.

Tc1/Th1, Tc2/Th2, and Tc17/Th17 cells and cytokines

No correlations were observed between any of these cell subsets and any measures of muscle strength, performance, or body composition. However, the relative frequency of CD8$^+$

Figure 3 CD197 expression is significantly reduced in people with cancer. CD197 mRNA expression in whole blood from people with (n = 11) and without (n = 9) cancer was quantified using real-time RT-PCR (A). CD197 and CD45RA protein expression was determined on a single western blot (B and C). Panel B shows the combined data from all samples in both groups. Panel C shows all samples from each group on the same gel. Groups are compared using the Student's *t*-test. Data shown are mean ± SEM. The *P* value is shown on panel A.
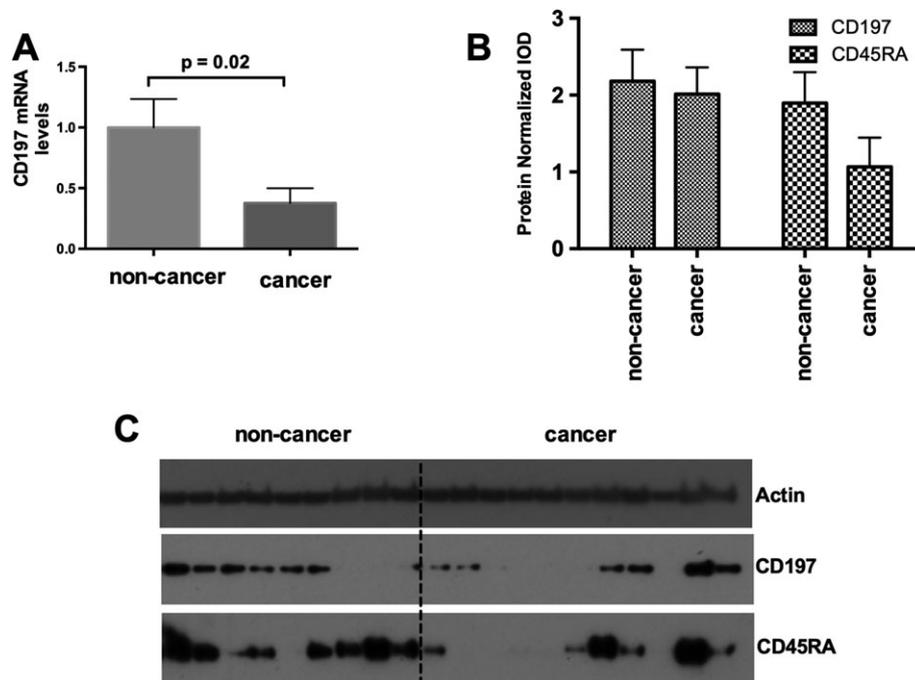

Figure 4 The frequency of CD95^{+} T cells and non-T cells correlates with lower body muscle strength in people with cancer. Correlations between relative frequency of CD8^{+} CD95^{+} T cells and stair climb power (SCP) (A) and knee extension strength (B). Correlations between the relative frequency of CD3^{−} peripheral blood mononuclear cell (PBMC) in people with cancer that express CD95 and either SCP (C) or knee extension strength (D). Spearman correlation (r) and statistical significance (P) are shown on panels A–D. Each symbol represents a single participant.
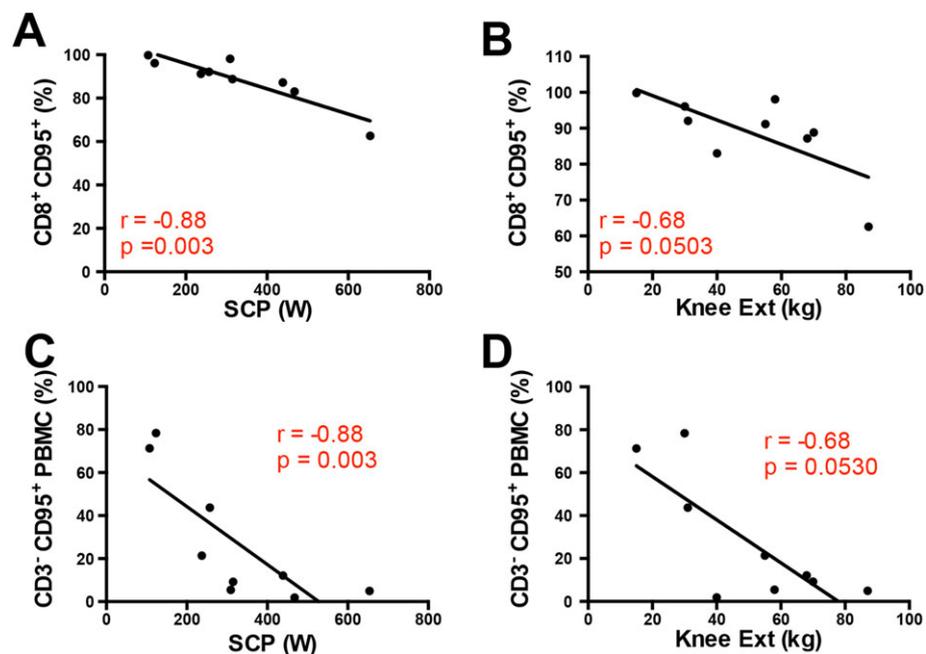
Figure 5 The level of CD95 gene expression in whole blood correlates with muscle strength. CD95 mRNA in whole blood (A) and CD95 and caspase 3 protein expression in peripheral blood mononuclear cell (B and C) was quantified using real-time RT-PCR and western blot from people with (n = 11) and without (n = 9) cancer. Panel B shows the combined data from all samples in both groups. Panel C shows all samples from each group on the same gel. Groups were compared using the Student's t-test. Data shown are mean ± SEM. The P value is shown on panel A. Spearman correlations (r) in the group with cancer are shown between the levels of CD95 mRNA expression in whole blood, quantified by real-time RT-PCR, and either knee extension strength (D) or stair climb power (SCP) (E).
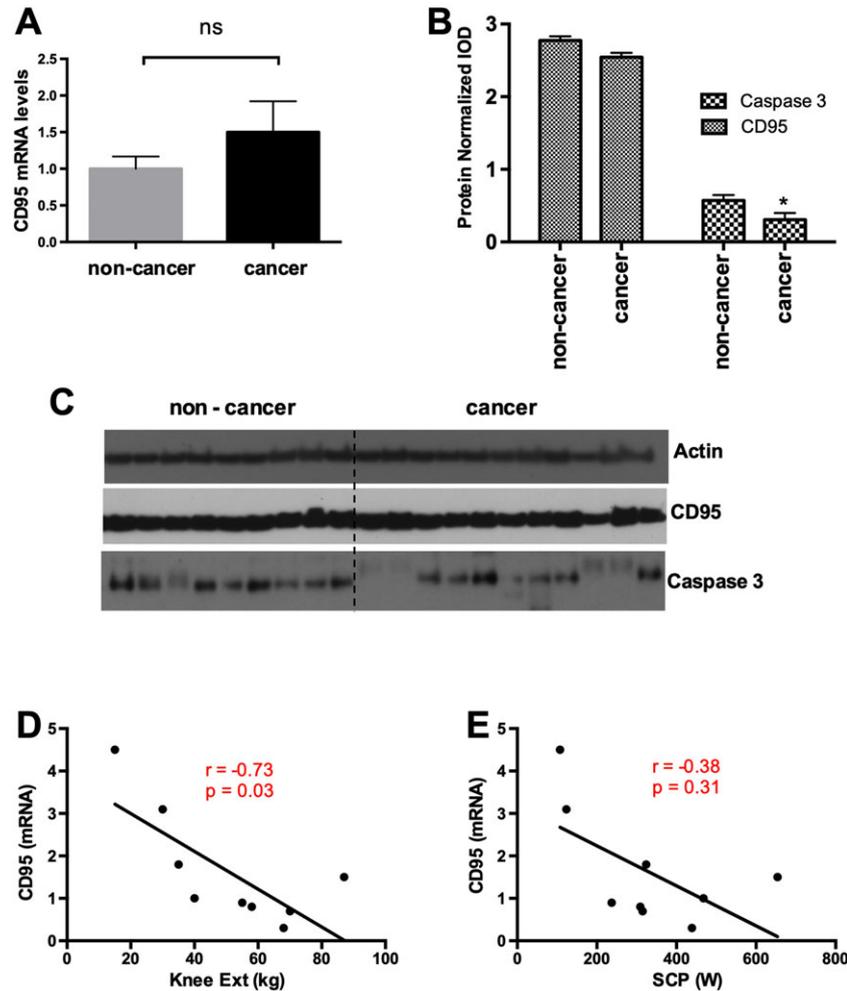
Figure 6 Treg cell frequency correlates with a low lean mass index (LMI) in people with cancer. Correlations between the relative frequency of Treg (A) with LMI in people with cancer. The LMI shown is the LMI in kg/m^2 × 1000. Spearman correlation (r) and statistical significance (P) are shown. Panel B, Treg cell frequency, is compared between groups using the Student's t-test. Data shown are mean ± SEM.
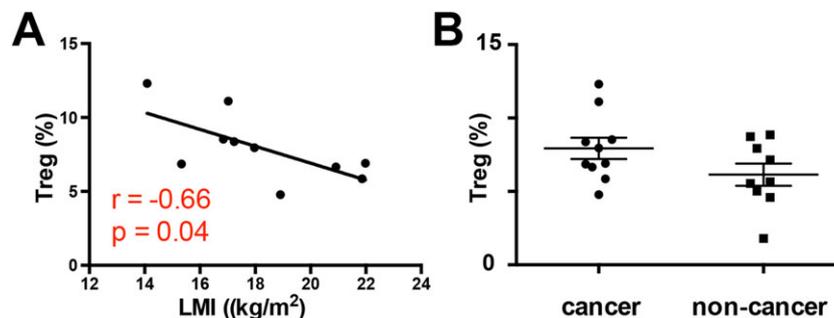
cells expressing IL-2, a cytokine produced and secreted during T cell activation,[52] correlates significantly with LMI (*Figure* 7A) and shows a trend with BMI (*Figure* 7B) in the group with cancer. The frequency of IL-2^{+} CD8^{+} T cells is not significantly different between people with or without cancer (*Figure* 7C). Schematics to show the gating strategy used to identify IL-2-expressing CD8^{+} T cells, and a representative example of the cell subset, is shown in Supporting Information, *Figure S4*. No correlations were found between either FMI or BMD and cell subset frequencies.

Immune correlations with functional impairment

As expected, the Karnofsky index is significantly lower (*Figure* 8A) and the ECOG-PS significantly higher (*Figure* 8B) in the cancer group than in the non-cancer control group. None of the cell subsets tested correlated with the level of functional impairment using either of these tests. However, the level of expression of CD4 mRNA in whole blood correlates significantly and inversely with the Karnofsky index (*Figure* 8C) but not with the ECOG-PS (*Figure* 8D). The expression of no

Figure 7 Interleukin (IL) 2-expressing CD8^{+} T cells and body composition. Correlations between the relative frequency IL-2 expressing CD8^{+} T cells and either lean mass index (LMI) (A) or body mass index (BMI) (B) in people with cancer (*n* = 8). The LMI shown is the LMI in kg/m^{2} × 1000. Spearman correlation (*r*) and statistical significance (*P*) are shown on panels A and B. Each symbol represents a single participant. Panel C, IL-2^{+} CD8^{+} cell subset frequency, is compared between groups with (*n* = 8) and without (*n* = 8) cancer using the Student's *t*-test. Data shown are mean ± SEM.
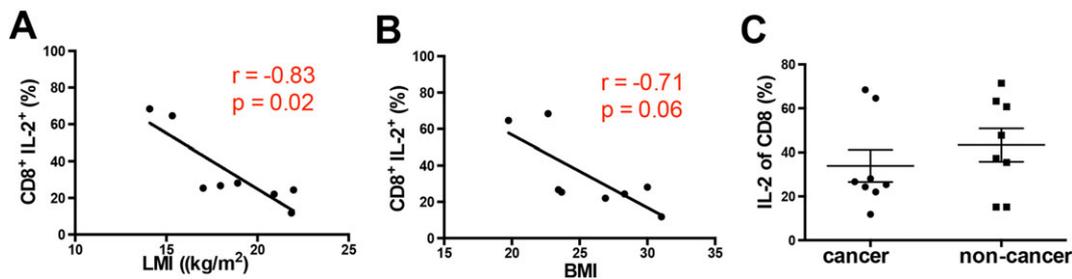
Figure 8 Immune correlations with the Karnofsky Performance Scale Index and ECOG-PS. The level of CD4 expression in whole blood from people with (*n* = 11) and without (*n* = 9) cancer was determined by real-time RT-PCR. In the same subjects, performance was determined using both the Karnofsky index and the ECOG-PS. Panels A and B compare the Karnofsky index (A) and ECOG-PS (B) in people with and without cancer. Correlations between CD4 levels and either the Karnofsky index (C) or the ECOG-PS (D) are shown for people with cancer. In panels A and B, groups are compared using the Student's *t*-test. Data shown are mean ± SEM. The *P* value is shown on each panel. Spearman correlation (*r*) and statistical significance (*P*) are shown on panels C and D for the cancer group. Data are not significant for the non-cancer group.
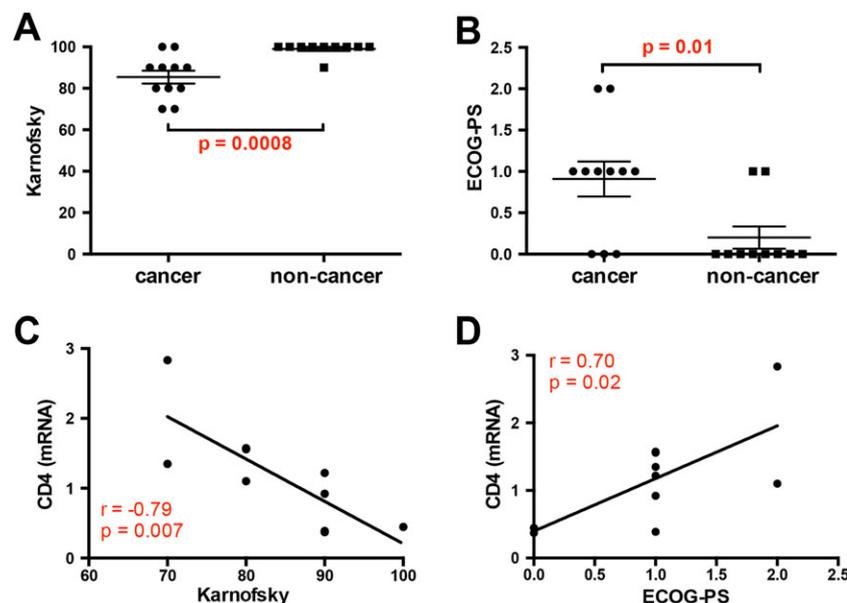
other factor, whether measured by mRNA or protein, correlates with either of these functional tests.

Discussion

This pilot study identifies several candidate naïve, memory, and regulatory T cell and non-T cell populations that strongly correlate with levels of muscle strength, performance, and body composition. These correlations exist in patients with cancer, but not in people without cancer, suggesting that cancer modulates muscle health and immune T cells in a way that is linked. Unexpectedly, the T cell and non-T cell populations identified do not correlate uniformly with all measurements of muscle health. Instead, naïve and memory T cell frequencies correlate with HGS and chest press strength (both upper body); the frequency of CD95-expressing cells correlates with knee extension capacity and SCP (both lower body); and regulatory cell frequencies correlate with body composition, specifically BMI and LMI.

The finding that the significant correlations between immune cell frequencies and measures of muscle health did not exist in the group without cancer, even though the cell frequencies and levels of muscle health (with the notable exception of HGS) are not different between groups, poses a potential paradox. We suggest that the levels of muscle strength, performance and mass, and immune profiles in healthy and unhealthy people are so variable that the standard deviation between groups is too high to result in a significant difference. In contrast, variability in these measurements within a group is an advantage to perform correlative analysis. Because we find significant correlations in the group with cancer, but not in the group without cancer, it appears that cancer might be a driving force in modifying muscle health and the immune system through a mechanism that is linked.

In a successful immune response to antigen, naïve T cells become activated, divide to increase their number and frequency, and differentiate to become EM cells with the capacity to kill the target cell, whether to eradicate a pathogen[53,54] or destroy cancer cells.[55] In response to cancer, if such immune surveillance fails, cancer cells divide to create a detectable mass of cells, a tumour. The strong correlations that we have identified between a high relative frequency of EM CD8^{+} T cells and stronger HGS and chest press capacity in patients with cancer suggest that CD8^{+} T cell surveillance might continue during cancer recurrence in some patients. Some control of tumour burden might then reduce the detrimental effect that tumour growth has on muscle function resulting in stronger muscle measures.[13] Our data do not address whether the CD8^{+} EM cells in cancer participants with greater muscle strength contain cancer-specific cells. However, these data, together with the published associations between HGS and overall survival,[10,56] are consistent with the hypothesis that a stronger handgrip is associated with longer overall survival in people with a more effective anti-cancer CD8^{+} T cell response. The correlation between lower LMI and higher frequency of CD8^{+} IL-2^{+} T cells might at first seem to contrast with the data showing that a high frequency of EM correlates with greater muscle strength, because IL-2 is a marker of T cell activation.[52] Although CD8^{+} IL-2^{+} cells can be found within the CD8^{+} EM cell subset and *vice versa*, there is also a non-overlapping population that differs in phenotype and function. We predict that it is the non-overlapping population that relates to either muscle strength (EM) or LMI (IL-2^{+}) in people with cancer.

Recent thymic emigrants are the most recently exported T cells from the thymus, hence their name, RTEs.[39,40] RTEs that have not undergone cell division maintain a diverse TCR repertoire, a critical feature of a T cell population that allows them to respond to a diverse set of antigens, including cancer antigens.[57] Our finding that there is a positive correlation between CD8^{+} RTE and HGS and chest press might reflect the capacity of the immune system to mount new anti-cancer immune responses.

Expression of CD197, also called CCR7, by naïve and CM T cells allows their entry into and recirculation through secondary lymphoid organs where they encounter antigen.[58] The finding that the relative frequency of naïve T cells correlates with the level of muscle strength fits with the scenario that people with cancer with the most naïve cells, particularly RTE, are most likely to mount a T cell response to cancer antigen because naïve cells have the most diverse TCR repertoire, and of the naïve T cell population, RTEs are the most diverse.[57] On the other hand, CM are less useful because their TCR diversity is not as high as RTE, and, unlike their EM counterparts, they cannot migrate out of the circulation and into the tumour microenvironment.[59]

The two main regulatory T cell subsets that have been associated with disease progression in people with cancer are Foxp3^{+} regulatory cells, generally referred to as Treg cells, and Th2 cells, a subset of effector T cells that is dependent on IL-4 for its development and function. Both Treg[4] and Th2 cells[6] are anti-inflammatory, and their capacity to inhibit inflammatory T cell responses, including anti-cancer effector T cell responses, might explain their association with poor prognosis in people with cancer. We have recently described a new Th2-type CD4^{+} T cell population that expresses CD25 and a high density of CD127 (CD25^{+} CD127^{hi}) that correlates with slow disease progression in children newly diagnosed with type 1 diabetes.[41,42,60] Although the mechanism of action of CD4^{+} CD25^{+} CD127^{hi} cells is not known, it is possible that they also play a role in limiting inflammatory T cell responses in people with cancer. Taken together, these data might predict that poor muscle function in people with cancer would correlate with a high relative frequency of Treg, including activated Treg and CD4^{+} CD25^{+} CD127^{hi} cells. However, an argument against this prediction stems from the well-established finding that pro-inflammatory cytokines are

elevated in some people with cancer who have compromised muscle mass and strength[61–64] and that anti-inflammatory cytokines, including IL-4 and IL-10, can have protective properties.[65,66] Our data showing correlations between a higher frequency of Treg, but not Th2 cells or CD25^{+} CD127^{hi} cells, with lower LMI in people with cancer are consistent with the hypothesis that enhanced T cell regulation via Treg inhibits the anti-cancer EM T cell response leading to a higher tumour burden, disease progression, and the associated negative effects on body composition.

Previously, investigators have described that an increase in metabolic burden on the body resulting from a non-specific increase in overall immune activity might cause muscle loss.[14] In our study, we found that an increase in relative frequency of CD8^{+} IL-2^{+} T cells, a subset of activated T cells, correlates with a reduction in lean mass. It is possible that the increase in frequency of CD8^{+} IL-2^{+} cells reflects an increase in absolute numbers of these cells and that the resulting increase in metabolic demand caused by larger numbers of activated T cells might result in a reduction in muscle mass. In future studies, measuring absolute numbers of CD8^{+} IL-2^{+} T cells in whole blood might shed mechanistic insight into potential causal links.

CD197 is expressed by B cells, monocytes, and dendritic cells as well as T cells[67–69] and is the receptor for chemokines, CCL19 and CCL21.[70,71] Both of these chemokines are secreted by cancer cells.[72] Similar to the data with T cells, the correlations between CD197-expressing non-T cells are either positive or negative depending on whether the cells co-express CD45RA (naïve, good muscle function) or not (memory, poor muscle function). Signalling via CD197 after ligation with either CCL19 or CCL21 controls chemotaxis and cell migration speed.[73] The association between fewer CD197^{+} naïve cells and lower muscle strength might suggest that cancer specifically inhibits CD197 expression on naïve cells to prevent their migration to secondary lymphoid organs where they would be primed for an anti-cancer T cell response.

CD95 is widely recognized as Fas, a multifunctional receptor that can provide either a pro-death[74,75] or a pro-survival signal[76,77] depending on the environment. The significant lower expression of caspase 3 in the PBMC from people with cancer compared with non-cancer control subjects suggests that, in this study, CD95 expression is associated with survival and not apoptosis. The correlations found between high frequency of CD95 expressing CD8^{+} T cells and non-T cells with reduced SCP and knee extension strength in people with cancer suggest a non-specific effect of cancer in simultaneously promoting the survival of circulating cells that express CD95 as muscle function is lost.

As part of the design of this study, we predicted that if immune cell measures could be used to assess muscle function and performance, they might also correlate with perceived functional impairment. The level of CD4 gene expression measured in whole blood, but not the frequency of CD4^{+} T cells, or any other T cell or non-T cell population measured,

significantly correlated with both the Karnofsky Performance Scale Index.[47] These data might suggest that an increase in turnover of CD4 expression indicates weak immunity to cancer antigen.

Previously published results have shown changes in the frequency of several T cell subsets in people with cancer, including CD8^{+} and CD4^{+} T cells, Treg cells,[1] and pro-inflammatory Th17 and Th22 cells.[2] Other studies have reported a reduction in B cells[1] in people with cancer. Correlations between prognosis and the frequency of CD8^{+} naïve and memory T cells[3] and Treg[4] have also been reported. Our data extend these findings by showing that naïve, memory, and regulatory T cell subsets that were previously associated with disease progression in people with cancer[1–4] also correlate with independent predictors of cancer progression, specifically measures of muscle function.

In addition, we have identified several new cell populations not previously recognized as relevant to either cancer progression or muscle function. Most notably, the data presented might shed light on the mechanism by which levels of muscle strength and mass might predict cancer recurrence and overall survival.

Study limitations and strengths: (i) This is a pilot study, and as such, the number of participants in each group is small, 11 in the cancer group and nine in the non-cancer group. Although we are encouraged by the strength of the data, a second study with a larger and different cohort of participants will be necessary to validate our findings. (ii) Although the focus of the study was to determine whether there were significant correlations between anti-cancer T cell profiles and muscle strength, we measured and tested over 40 T cell and non-T cell populations. Because this is an exploratory study, no adjustments were made for the multiple hypotheses conducted. A follow-up confirmatory study is planned. Nevertheless, if there were no relationships between these variables, we would expect two cell subsets out of 40 cell subsets conducted at the 5% significance level to correlate with muscle strength, mass, and performance; in fact, 10 of the 40 cell subsets tested were statistically significant at the 5% level, a result that is itself statistically significant and indicates that at least some of these relationships are likely to be confirmed in the follow-up study. (iii) All participants in the cancer group were diagnosed with cancer in the GI tract, but the types and stages of cancer are highly variable, as described in *Table* 2. Such heterogeneity is often seen as a disadvantage in the design of a clinical study because commonalities are often not evident. However, the strong correlations that we report between muscle health and immune cell subset frequency in this population of people might suggest that the links found are related to biology that is common to all GI cancers. (iv) Our data identify correlations between immune cell subsets and measures of muscle health. The data do not address whether there is a causal relationship between muscle health and immune cell frequency or function. Further research is

necessary to understand the nature of the relationship between skeletal muscle, T cells, and other immune cell markers. (v) To accurately quantify absolute numbers of cell populations in blood, cells should be counted per volume of whole blood before the blood is processed. In this study, relative cell yield between participants cell numbers was determined after PBMCs were isolated.

In conclusion, our pilot study has been successful in identifying several candidate immune cell populations that are associated with muscle function in people with cancer. A follow-up study is planned with a larger study cohort to test whether these data are reproducible. If validated, our data might suggest that muscle function can provide surrogate measures of the level and type of immune surveillance. There are several mechanisms by which the cancer itself might modulate immune surveillance including the secretion of factors, CCL19 and CCL21, to modulate the frequency of CD197 expressing naïve and CM cells in an attempt to limit the frequency of EM T cells to the cancer site, and the expression of CD95 to modulate cell survival. Although the mechanism(s) by which cancer, immune cells, and muscle health are linked are yet to be determined, these data are consistent with the hypothesis that previously published associations between muscle strength and mass with cancer recurrence and survival can be explained if linked to the level of ongoing immune surveillance.

Acknowledgements

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of Antibodies Used
Figure S1. CD8+ RTE, CM and EM
Figure S2. CD8+ CD95+ and CD4+ CD95+ T cells
Figure S3. Regulatory T cells (Treg)
Figure S5. IL-2 expressing CD8+ T cells

Conflict of interest

None declared.

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