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A Novel Role for CD4⁺ T Cells in the Control of Cachexia¹

Zhuangzhi Wang,² Chunfang Zhao,² Rosa Moya,² and Joanna D. Davies^{2,3}

Cachexia is the dramatic weight loss and muscle atrophy seen in chronic disease states, including autoimmunity, cancer, and infection, and is often associated with lymphopenia. We have previously shown that CD4⁺ T cells that express the lowest density of CD44 (CD4⁺CD44^{v.low}) are significantly reduced in diabetic NOD mice that are cachexic compared with diabetic mice that are not cachexic. Using this model, and a model of cancer cachexia, we test the hypothesis that CD4⁺CD44^{v.low} cells play an active role in protecting the host from cachexia. CD4⁺CD44^{v.low} cells, but not CD4⁺ cells depleted of CD44^{v.low} cells, delay the onset of wasting when infused into either diabetic or prediabetic NOD recipients. However, no significant effect on the severity of diabetes was detected. In a model of cancer cachexia, they significantly reduce muscle atrophy, and inhibit muscle protein loss and DNA loss, even when given after the onset of cachexia. Protection from wasting and muscle atrophy by CD4⁺CD44^{v.low} cells is associated with protection from lymphopenia. These data suggest, for the first time, a role for an immune cell subset in protection from cachexia, and further suggest that the mechanism of protection is independent of protection from autoimmunity. *The Journal of Immunology*, 2008, 181: 4676–4684.

Cachexia, characterized by dramatic weight loss and muscle atrophy, is a consequence of a number of chronic disorders, including AIDS (1), aging (2–4), and type 1 diabetes (T1D)⁴ (5). An association between immunodeficiency and cachexia (6–10) has led us to speculate that a deficiency in the immune system might play a direct role in the development of cachexia, and that correcting or inhibiting the immune deficiency might allow protection.

A deficiency in CD4⁺ T cells that express a low density of the cell surface marker CD44 (CD44^{low}) has been associated with aging (11–13) and with the development of spontaneous tumors (14, 15). In addition, we have recently shown that a subset of CD4⁺CD44^{low} cells, defined by their expression of the lowest density of CD44 (CD4⁺CD44^{v.low}), is depleted in diabetic mice at the onset of cachexia, but not in diabetic mice that are not cachexic (16). These data implicate the CD4⁺CD44^{v.low} T cell subset as a hypothetical candidate for modulating the development of cachexia. CD44 is one of the well-established cell surface markers used to distinguish Ag-inexperienced (naive) from Ag-experienced (memory) CD4⁺ T cells in the mouse. Thus, naive CD4⁺ T cells express CD44^{low} and a high density of CD62L (CD62L^{high}), whereas memory cells express CD44 at a high density (CD44^{high}) (17, 18). Naive cells also express a high density of CD45RB (CD45RB^{high}) (19–21). By these criteria, the CD4⁺CD44^{v.low} T

cell subset, which is deficient in diabetic mice that are cachexic, is a naive CD4⁺ T cell.

Autoimmune destruction of pancreatic β cells in T1D results in low insulin production and high blood glucose levels (BGL) (22, 23). Without insulin treatment, individuals with T1D develop cachexia (5, 24). We have shown that wasting in the NOD mouse, the well-established mouse model for T1D (25, 26), is also due to cachexia, with a dramatic loss of skeletal muscle weight, significant muscle protein loss, and activation of the ubiquitin proteasome pathway (16). In addition, muscle atrophy in the NOD mouse is associated with the presence of DNA fragmentation and a significant loss in muscle DNA content, suggesting the possibility that T1D cachexia, like some models of cancer cachexia, involves apoptosis (27–29).

Using the NOD mouse model for T1D cachexia, we find that infusion of highly purified CD4⁺CD44^{v.low} cells, but not CD4⁺ cells that are depleted of CD44^{v.low} cells, into prediabetic NOD mice significantly delays the onset of wasting and muscle atrophy, but no effect on the severity of diabetes was detected. CD4⁺CD44^{v.low} cells also inhibit muscle atrophy when infused into NOD mice that already have diabetes. That the mechanism of protection induced by CD4⁺CD44^{v.low} cells is independent of an effect on T1D is further suggested by the finding that CD4⁺CD44^{v.low} cells also inhibit muscle atrophy, including muscle protein and DNA loss, in a C57BL/6 mouse strain model for cancer cachexia. In addition, infusion of CD4⁺CD44^{v.low} cells, but not CD4⁺ cells depleted of CD44^{v.low} cells, results in significant inhibition of Lewis lung carcinoma cell (LL2)-induced CD4⁺ T cell lymphopenia in the cancer cachexia model, suggesting that they might modulate cachexia by a mechanism that involves protection from CD4⁺ T cell lymphopenia. To our knowledge, this is the first report that indicates a role for CD4⁺ T cells in protecting the host from muscle atrophy, and suggests a novel role for a CD4⁺CD44^{v.low} cell subset, and the maintenance of immune homeostasis, in controlling cachexia.

Torrey Pines Institute for Molecular Studies, San Diego, CA 92121

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² Current address: Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, CA 92121.

³ Address correspondence and reprint requests to Dr. Joanna D. Davies, Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, CA 92121. E-mail address: jdavies@tpims.org

⁴ Abbreviations used in this paper: T1D, type 1 diabetes; BGL, blood glucose level; int, intermediate; LL2, Lewis lung carcinoma cell.

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to institutional guidelines and approved by the Institutional Animal Care and Use Committee.

Assessment of diabetes

Every 2 wk for the duration of the experiment, BGL were tested using a one-step Bayer Glucometer Elite (Bayer). Mice were considered diabetic when the BGL were >300 mg/dL over two consecutive readings.

LL2-induced cachexia

LL2 is a cell line derived from the Lewis lung carcinoma. C57BL/6 mice were injected with 5×10^5 LL2 in the left thigh. By day 7 post-LL2 injection, a small nodule can be detected in the thigh by palpitation. Mice were terminated and tissues were removed on or before day 28 post-LL2 injection, as indicated for each experiment.

Assessment of wasting

NOD mice were weighed once per week for the duration of the experiment. C57BL/6 mice injected with LL2 cells were weighed once per week for the first 2 wk post-LL2 injection and then daily. Mice were considered wasting when their body weight was 20% less than at the beginning of the experiment. Weight loss in excess of 20% was associated with morbidity and mortality, and therefore, wasting mice were sacrificed and tissues were taken for analysis within 24 h of wasting assessment, or before, as indicated in each experiment.

Cell purification and transfer

Spleen cells from either 2- to 4-mo-old mice were prepared for single-cell suspensions. RBC were removed with lysing buffer (Sigma-Aldrich), and the remaining spleen cells were resuspended in PBS with 1% FBS (Serologicals). Splenocytes were labeled with an allophycocyanin-conjugated CD4-specific mAb and PE-conjugated CD44-specific mAb and CD4⁺CD44^{v.low} cells, and CD4⁺ cells depleted of CD4⁺CD44^{v.low} cells, were sorted under high speed on a FACSVantage SE with TurboSort (BD Immunocytometry Systems). The CD4⁺CD44^{v.low} population was defined as the CD4⁺ cells that stain the weakest for CD44, and was typically 3–5% of the total CD4⁺ cells in nondiabetic NOD mice (16) and 1–2% of the total CD4⁺ cells in untreated C57BL/6 mice. To avoid contamination with CD4⁺CD44^{int} cells, only the weakest staining 2% of CD4⁺CD44^{v.low} cells in NOD and 0.8% in C57BL/6, and the brightest staining 80% of CD4⁺ cells depleted of CD4⁺CD44^{v.low} cells were collected for the experiments described in this study. All cell populations were sampled and analyzed using a FACSCalibur to confirm the purity of the sorted populations. Sorted CD4⁺ cell populations were greater than 98% CD4⁺ (data not shown). Cells were washed once in PBS after sorting and before i.p. injection into syngeneic recipients.

Histology and histologic assessment

Mice were sacrificed at the indicated times, and the pancreas was removed and immediately placed in 10% neutral buffered formalin to be fixed. After 24 h, the pancreata were embedded in paraffin, and 4- μ m sections were cut. Sections were tested for the presence of insulin-producing β cells by immunohistochemistry (30). Sections were stained for insulin with guinea pig anti-porcine insulin Ab (DakoCytomation) using the indirect immunoperoxidase method. Sections were hydrated and blocked with 10% normal goat serum (Vector Laboratories). The sections were then incubated overnight in primary Ab and treated with biotinylated secondary Ab (Vector Laboratories), followed by an avidin/biotinylated enzyme complex (Vector Laboratories). Slides were incubated in the dark with the enzyme substrate, 0.05% 3,3'-diaminobenzidine (Sigma-Aldrich) and counterstained, dehydrated, and mounted with Permount (Fisher Scientific). Insulin-positive areas were stained brown. The insulin-positive area was accurately scored in pixels by measuring the brown coloration (insulin-positive area after insulin-specific staining) in each section of each pancreas using a Zeiss Axiovision camera and acquisition software (Carl Zeiss) and KS300 analyzing system (Carl Zeiss). For each pancreas, the islet area from three sections 20 μ m apart was analyzed and the mean was calculated.

Skeletal muscle protein isolation and quantitation

The left and right anterior and lateral thigh muscles were isolated, weighed, then individually wrapped in autoclaved aluminum foil and stored at -80°C until analyzed. The packed muscle was immersed in liquid nitrogen and ground with mortar and pestle. The powdered tissue was transferred into 1 ml of ice-cold homogenization buffer (0.01 M Tris, 2 mM EDTA, 0.15 M NaCl, 0.012 M Brij 96, 2.22 mM Nonidet P-40, 0.025 mM leupeptin, 0.025 mM aprotinin, and 0.025 mM 4-(2-aminoethyl)benzenesul-

phonyl fluoride hydrochloride) and homogenized with an electronic pellet pestle. The homogenates were incubated for 30 min at 4°C , and centrifuged at $14,000 \times g$ for 10 min at 4°C . Supernatants were thawed and diluted 1/800 in distilled H₂O on ice. Soluble protein concentration was determined by mixing 160 ml of the diluted sample with 40 ml of Bio-Rad dye reagent (Bio-Rad) in a 96-well plate using BSA as the protein standard. Using this information, the weight of soluble protein in each whole muscle was calculated. Supernatant measurements were performed at least in duplicate. The plates were incubated for 10 min at room temperature and read at 595 nm on a microplate reader (Molecular Devices).

Skeletal muscle DNA isolation and quantitation

The lateral and anterior thigh muscles were excised from both hind legs of each mouse and weighed. Tissue samples (50 mg) were minced and then lysed in a 6 M guanidinium chloride buffer containing proteinase K (40 μ g/ml) at 55°C for 2–4 h, and then treated briefly with DNase-free RNase following the DNeasy protocol (Qiagen). Aliquots of DNA were diluted in 1 M urea for total DNA concentration measurements using a fluorometric DNA assay (31) with Hoechst dye 33258 (Bio-Rad), and the weight of DNA in each whole muscle was calculated.

Cell subset analysis

Single cell-suspensions of lymph nodes (cervical, mesenteric, inguinal, para-aortic) were labeled with an allophycocyanin-conjugated CD4-specific mAb (RM4-5) and PE-conjugated CD44-specific mAb (IM7). Allophycocyanin-conjugated rat IgG2a and PE-conjugated rat IgG2b were used as isotype controls. All cell populations were sampled and analyzed using a FACSCalibur with CellQuest version 3.3 software (BD Immunocytometry Systems), and the percentage and total number of CD4⁺CD44^{v.low} (lowest fluorescent intensity peak for CD44 expression), CD4⁺CD44^{int} (middle fluorescent intensity peak), and CD4⁺CD44^{high} (highest fluorescent intensity peak), as described previously (16), were determined. All mAbs and isotype controls were purchased from BD Pharmingen.

Statistical analysis

The significance of the effect of CD4⁺CD44^{v.low} cells on protection from cachexia on transfer into NOD recipients was assessed using the log rank (Mantel-Cox) test. The significance of the effect of CD4⁺CD44^{v.low} cells on insulin-secreting cells in the pancreas, the effect of LL2 cell treatment on CD4⁺ T cell lymphopenia, and the effect of CD4⁺CD44^{v.low} cells on inhibition of CD4⁺ T cell subset lymphopenia was determined using the Mann-Whitney test. Skeletal muscle weight loss, skeletal muscle protein, and DNA content in cachexic animals were determined using Student's *t* test. A *p* value equal to or less than 0.05 is considered significant for all tests.

Results

Onset of wasting and diabetes in NOD mice

Fifteen NOD female mice were monitored for diabetes and wasting from the age of 10 wk. Ten of the mice were diabetic by 28 wk of age (Fig. 1*a*), and seven of these diabetic mice were wasting by 7 wk postdiabetes onset (Fig. 1*b*). As we have previously shown (16), none of the five nondiabetic mice became wasting during the time course of the experiment, and the diabetic mice that were wasting did not lose weight until after the onset of diabetes.

CD4⁺CD44^{v.low} cells decrease both the incidence and the severity of wasting, but not diabetes

We have previously shown that wasting in the NOD mouse is due to cachexia, and that the onset of cachexia in the diabetic NOD mouse is associated with a significant loss of CD4⁺CD44^{v.low} cells in the spleen and lymph nodes (16). To test the hypothesis that diabetic mice are protected from cachexia by the presence of CD4⁺CD44^{v.low} cells, we first tested the ability of these cells to inhibit the onset of diabetes and wasting in the NOD mouse. Prediabetic female NOD mice were infused with either highly purified CD4⁺CD44^{v.low} cells or no cells, and then monitored for diabetes and wasting. CD4⁺CD44^{v.low} cells significantly decrease the rate of onset and the incidence of wasting compared with untreated NOD mice (Fig. 2*a*; 67% wasting in untreated compared with 30% wasting in CD4⁺CD44^{v.low} cell treated; *p* = 0.05). In contrast, the

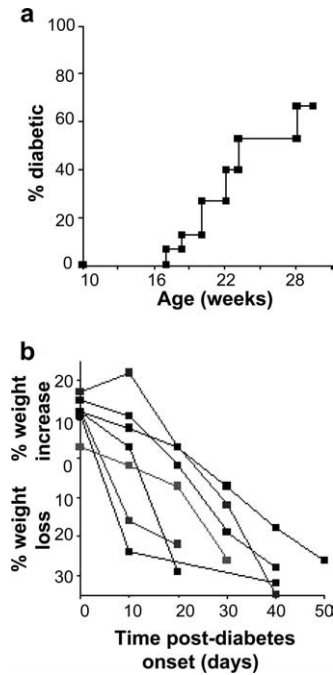


FIGURE 1. Onset of diabetes and wasting in NOD mice. A group of NOD female mice ($n = 15$) was monitored for the development of diabetes and wasting. *a*, Shows the incidence of diabetes onset with age. *b*, Shows body weight increase and loss (as a percentage of body weight at 10 wk of age), for each of seven diabetic mice. Percentage of body weight change is shown from the time of diabetes onset to the time taken to lose 20% original body weight. The data are representative of two experiments.

infusion of CD4⁺CD44^{v.low} cells did not inhibit either the onset or incidence of diabetes (Fig. 2*b*; 75% diabetic in untreated compared with 80% diabetic in CD4⁺CD44^{v.low} cell treated). When the total body weight of wasting and nonwasting diabetic mice (from Fig. 2*b*) was compared (Fig. 2*c*) and calculated as a percentage of the weight of each mouse at 10 wk of age (Fig. 2*d*), we found that

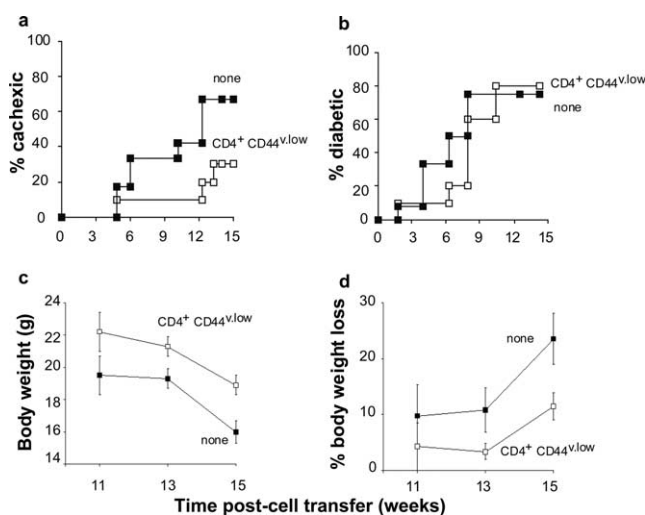


FIGURE 2. CD4⁺CD44^{v.low} cells decrease the incidence of wasting, but not diabetes. The incidence of wasting (*a*) and diabetes (*b*) was compared in female NOD mice injected at 10 wk of age with 2.5×10^5 sorted CD4⁺CD44^{v.low} cells ($n = 10$, □) isolated from spleens of 11-wk-old prediabetic NOD female donors, or with no cells ($n = 12$, ■). Body weight (*c*) and body weight as a percentage of weight of each mouse at 10 wk of age (*d*) show the severity of wasting in each group. Data in *c* and *d* are shown as mean \pm SEM. The data are representative of two experiments.

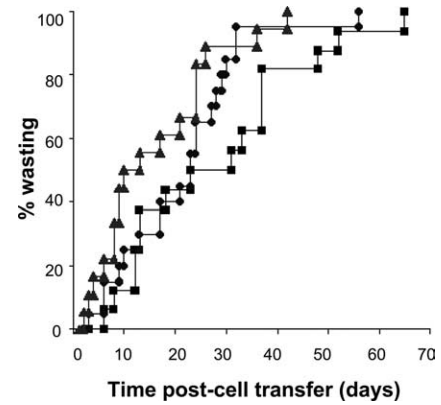


FIGURE 3. CD4⁺CD44^{v.low} cells significantly delay the onset of wasting when infused into diabetic NOD mice. Female NOD mice were monitored for the onset of diabetes. Within 1 wk after diabetes was diagnosed, diabetic mice were infused with either 5×10^5 sorted CD4⁺CD44^{v.low} cells isolated from prediabetic NOD mice ($n = 16$, ■), or an equal number of CD4⁺ cells depleted of CD44^{v.low} cells ($n = 20$, ●), also isolated from prediabetic NOD mice, or with no cells ($n = 18$, ▲). The incidence and time of onset of wasting are shown for each group and compared using the log rank Mantel-Cox test. $p = 0.02$ for untreated vs CD4⁺CD44^{v.low} cell treatment groups.

CD4⁺CD44^{v.low} cells significantly inhibited weight loss in diabetic mice ($p = 0.05$ at 13 wk post cell infusion, and $p = 0.03$ at 15 wk post cell infusion for both body weight loss and percentage of body weight loss), suggesting that CD4⁺CD44^{v.low} cells decrease the severity as well as the incidence of cachexia (Fig. 2, *c* and *d*).

In a separate experiment (Fig. 3), we compared the effect of infusing CD4⁺CD44^{v.low} cells into mice that were already diabetic, and compared this effect with the infusion of CD4⁺ T cells that were depleted of the CD4⁺CD44^{v.low} cell subset. Although CD4⁺CD44^{v.low} cells once again inhibited wasting compared with untreated NOD mice (triangles vs squares in Fig. 3, $p = 0.02$), CD4⁺ cells depleted of CD44^{v.low} cells did not (circles). Taken together, our data suggest a relationship between the CD4⁺CD44^{v.low} cell subset and modulation of cachexia, but not T1D.

CD4⁺CD44^{v.low} cells inhibit muscle atrophy

To confirm that the inhibition of wasting in diabetic NOD mice by CD4⁺CD44^{v.low} was associated with an inhibition of muscle atrophy, the skeletal muscle was isolated from the CD4⁺CD44^{v.low}-treated and untreated diabetic mice (described in Fig. 2), 15 wk post cell infusion, and weighed (Table I). The weight of skeletal muscle from diabetic NOD mice that received CD4⁺CD44^{v.low} cells was significantly greater than from untreated diabetic NOD mice (179 ± 27 mg in treated compared with 119 ± 9 mg in untreated; $p = 0.005$). Cachexia is associated with preferential loss of skeletal muscle mass (lean tissue mass). That is, in cachexia, the weight of skeletal muscle as a percentage of total body weight is less than it is in noncachexic mice. To determine whether CD4⁺CD44^{v.low} cells protected skeletal muscle preferentially or whether their effect was equivalent in skeletal muscle and the rest of the body weight, skeletal muscle was calculated as a percentage of total body weight for each mouse, and any ability of CD4⁺CD44^{v.low} cells to preferentially protect skeletal muscle was determined. In untreated nondiabetic mice, the skeletal muscle analyzed makes up $1.45 \pm 0.06\%$ of the total body weight (Table I). In contrast, in untreated diabetic mice, the same skeletal muscle is reduced to $0.76 \pm 0.02\%$ of the total body weight. If the

Table I. $CD4^+CD44^{v,low}$ cells inhibit skeletal muscle loss

Treatment ^a	Muscle Weight (mg) ^b	Body Weight (g) ^c	Muscle Weight as Percentage of Total Body Weight ^d
Diabetic ^e			
No treatment	119 ± 9 ^f	15.7 ± 1.5	0.76 ± 0.02
$CD4^+CD44^{v,low}$	179 ± 27	18.9 ± 2.0	0.95 ± 0.12
Nondiabetic			
No treatment	374 ± 20	26.3 ± 1.4	1.42 ± 0.06

^a Ten-week-old female NOD mice were either injected with 2.5×10^5 $CD4^+CD44^{v,low}$ cells isolated from 11-wk-old prediabetic NOD donors, or left untreated (no treatment), and monitored for the development of diabetes and wasting.

^b Diabetic mice were sacrificed at 25 wk of age, and skeletal muscle was isolated and weighed.

^c Mice were weighed at 25 wk of age.

^d Preferential skeletal muscle loss was determined by calculating muscle weight as a percentage of total body weight for each mouse. Untreated nondiabetic mice were used to generate baseline data for muscle weight, and muscle weight as a percentage of total body weight.

^e Diabetes is determined as described in *Materials and Methods*.

^f The values given are the mean ± SD for each group.

$CD4^+CD44^{v,low}$ cells inhibit skeletal muscle weight loss to the same extent as they protect wasting in the rest of the body, we would predict that percentage of total body weight that is skeletal muscle would not be significantly different from that seen in untreated mice ($0.76 \pm 0.02\%$). However, if $CD4^+CD44^{v,low}$ cells preferentially protect skeletal muscle, we would expect skeletal muscle in treated mice to make up a significantly greater percentage of total body weight than in untreated mice (greater than $0.76 \pm 0.02\%$). Data show that skeletal muscle weight in treated mice is $0.96 \pm 0.12\%$ of total body weight, and is significantly greater than that in untreated mice ($0.96 \pm 0.12\%$ compared with $0.76 \pm 0.02\%$, respectively, $p = 0.03$), suggesting that $CD4^+CD44^{v,low}$ cells preferentially inhibit muscle atrophy in diabetic NOD mice (Table I).

The effect of $CD4^+CD44^{v,low}$ cells on insulin-secreting β cells

Although data shown in Fig. 2 indicate that $CD4^+CD44^{v,low}$ cells do not affect the onset of diabetes, it does not exclude the

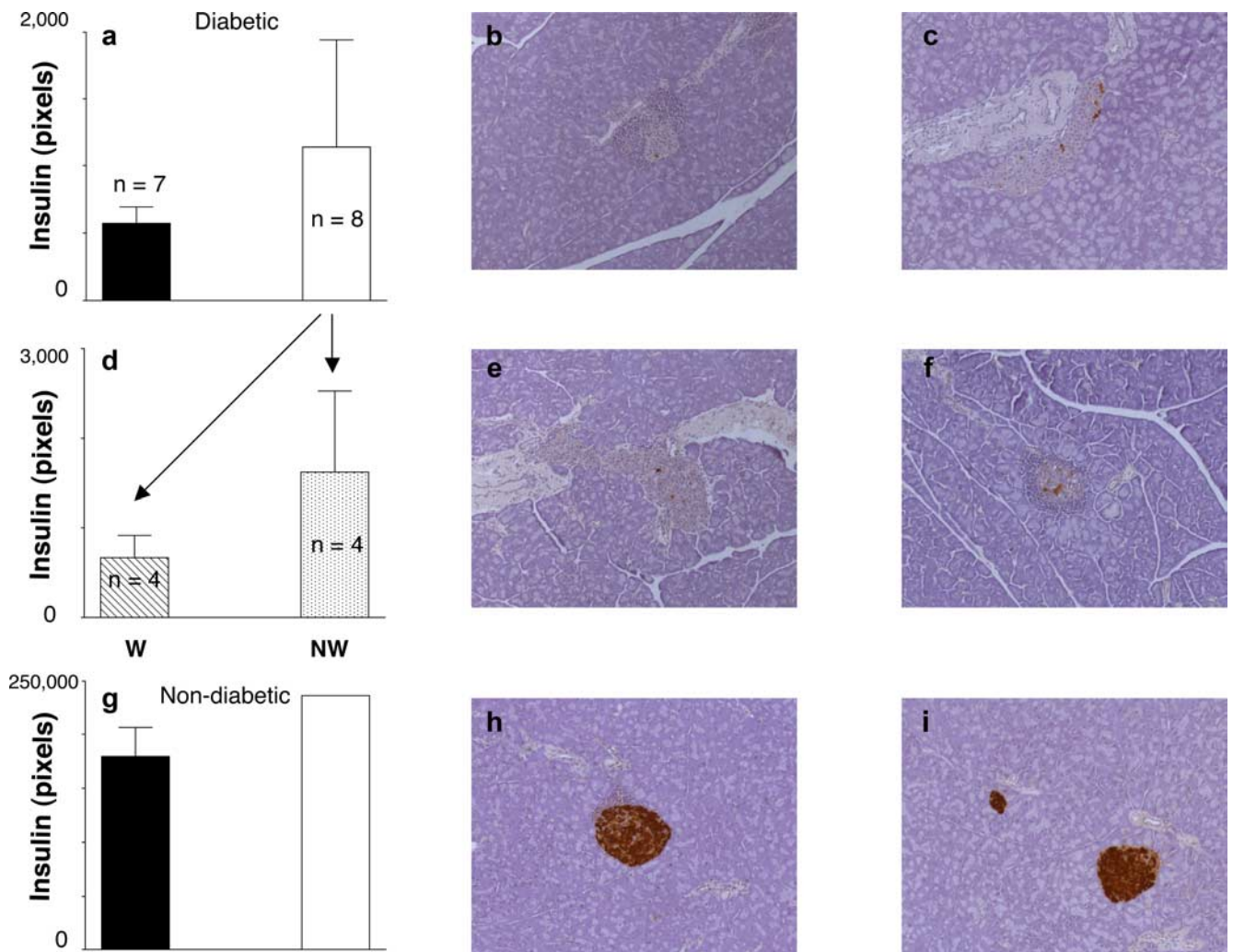


FIGURE 4. The effect of $CD4^+CD44^{v,low}$ cells on insulin-secreting β cells. The insulin-positive area in the pancreas was compared in female NOD mice injected at 9 wk of age with 2.5×10^5 $CD4^+CD44^{v,low}$ cells (treated, $n = 9$), or with no cells (untreated, $n = 9$). All mice were sacrificed at 15 wk after cell infusion, and the amount of insulin in the pancreas, measured in pixels, is shown in *a*, *d*, and *g*, as mean ± SD for each group. The representative sections of pancreas shown are stained for the presence of insulin (brown coloration). *a*, Shows the insulin area in diabetic untreated mice (7 of 9 mice were diabetic, and all diabetic mice were wasting) compared with diabetic treated mice (8 of 9 mice were diabetic, and 4 of the diabetic mice were also wasting). *b* and *c*, Representative sections of pancreata from diabetic untreated mice (*b*, diabetic and wasting) and diabetic treated (*c*, diabetic and wasting) mice. *d*, Compares the insulin area in pancreata from treated diabetic mice that were either wasting (W, $n = 4$) or not wasting (NW, $n = 4$). *e* and *f*, Representative sections of pancreata from treated wasting (*e*) and nonwasting (*f*) mice. *g*, Shows the insulin area in nondiabetic mice that were either untreated (shaded) or treated (open). *h* and *i*, Representative sections of pancreata from these nondiabetic untreated (*h*) and treated (*i*) mice.

possibility that they might inhibit the loss of insulin-secreting β cells to a level that is sufficient to modulate cachexia, but not diabetes. To address this issue, the effect of CD4⁺CD44^{v.low} cells on insulin-secreting β cell mass in the pancreas was tested. Prediabetic NOD mice were treated with and without CD4⁺CD44^{v.low} cells, and monitored for the development of diabetes and wasting. At 15 wk after cell infusion, the mice were sacrificed, and the pancreas was removed and assayed for the presence of insulin by immunohistochemistry. The relative amount of insulin in each pancreas was determined by measuring the insulin-positive area in pixels. The insulin area in the pancreas of diabetic mice that were untreated (7 of 9 mice were diabetic) was first compared with the insulin area in pancreas of diabetic mice that were treated with CD4⁺CD44^{v.low} cells (8 of 9 were diabetic; Fig. 4a). No significant difference was seen in the number of pixels of insulin measured in the pancreas of diabetic untreated (Fig. 4b) and diabetic treated (Fig. 4c) mice. Whereas all of the diabetic mice in the untreated group were also wasting by this time point, only four of eight diabetic mice in the treated group were wasting. However, although there was a trend that suggested an increase in insulin in the nonwasting group (Fig. 4d) compared with the wasting group (Fig. 4e), no significant difference was seen between these two groups. In addition, there is no difference (Fig. 4g) in the amount of insulin in the nondiabetic untreated (Fig. 4h) and treated (Fig. 4i) mice. The amount of insulin measured in pancreas from treated mice that were diabetic, but not wasting, was, at best, 1% of that measured in pancreas of nondiabetic treated and untreated mice (Fig. 4, g compared with d, respectively).

Cancer cachexia in C57BL/6 mice induced by LL2 is also associated with lymphopenia

Although the data to date suggest that the CD4⁺CD44^{v.low} cells inhibit cachexia by a mechanism that is independent of any effect on diabetes, we tested this possibility further using a model of cachexia that does not require diabetes as its primary disease. The LL2 model was chosen from a number of models of cancer cachexia because, unlike other models of cancer cachexia, the mechanism of cachexia induced by LL2, like that in the NOD mouse, involves skeletal muscle apoptosis in addition to activation of the ubiquitin proteasome pathway.

To determine whether cachexia in the LL2 model of cancer cachexia is also associated with lymphopenia, C57BL/6 mice were injected with LL2 cells and sacrificed at various times between 15 and 28 days afterward. Skeletal muscle was isolated and weighed. Consistent with published data, significant weight loss was seen in the skeletal muscle of LL2-treated mice by 25 days after injection compared with age-matched control mice (Fig. 5a). CD4⁺ T cell number in both spleens and lymph nodes was significantly reduced in cachexic mice compared with untreated control mice, indicating that the LL2 model for cancer cachexia is associated with CD4⁺ T cell lymphopenia ($p = 0.02$ for spleen and $p = 0.04$ for lymph node) (Fig. 5b).

CD4⁺CD44^{v.low} cells inhibit muscle atrophy in cancer cachexia

Having established the time of onset of cachexia in the LL2 model in our hands, we set out to determine whether infusion of CD4⁺CD44^{v.low} cells was also able to modulate cancer cachexia, as it does for diabetes-induced cachexia. C57BL/6 mice were injected with LL2, and on the same day infused with either CD4⁺CD44^{v.low} cells, CD4⁺ cells depleted of CD44^{v.low} cells, or no cells (Fig. 6). Skeletal muscle was removed and weighed on day 28 post-LL2 injection. Skeletal muscle weight was significantly greater in CD4⁺CD44^{v.low} cell-treated mice, compared with mice

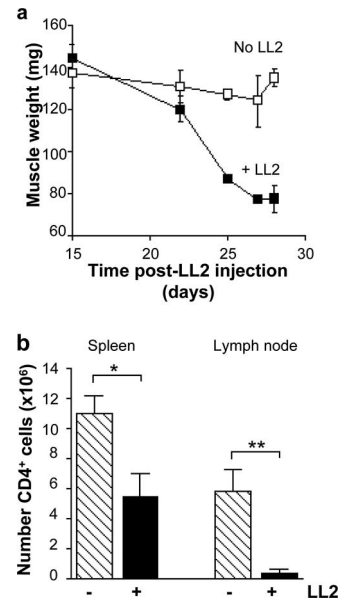


FIGURE 5. Cachexia in C57BL/6 mice induced by LL2 is also associated with lymphopenia. C57BL/6 mice were either injected with 5×10^5 LL2 cells in the left thigh or left untreated. Equal numbers of mice from each group were sacrificed at days 15 ($n = 4$), 22 ($n = 4$), 25 ($n = 2$), 27 ($n = 2$), and 28 ($n = 2$) post-LL2 injection, and skeletal muscle was isolated and immediately weighed. The data in *a* show the mean muscle weight \pm SD for each group at each time point. The number of CD4⁺ T cells in spleen and lymph nodes was determined on days 27 and 28 post-LL2 treatment and compared with age-matched untreated control mice. *b*, Shows mean \pm SD of pooled data from days 27 and 28; $n = 4$ per group. The data are representative of two separate experiments. The level of statistical significance is indicated as * for $p = 0.05$ – 0.01 and ** for $p = 0.009$ – 0.001 .

treated with either CD4⁺ cells that were depleted of CD44^{v.low} cells, or with no cells.

Similar data were obtained when CD4⁺CD44^{v.low} cells were infused 24 and 25 days post-LL2 injection, at a time when

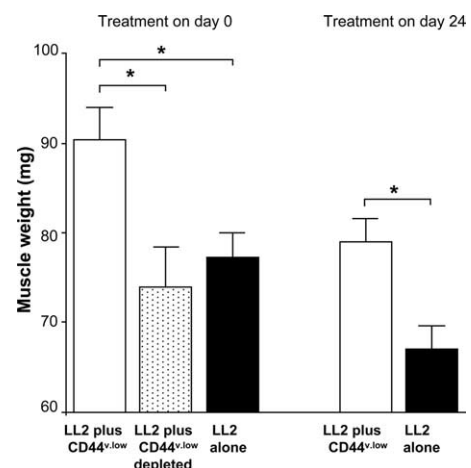


FIGURE 6. CD4⁺CD44^{v.low} cells inhibit muscle atrophy in cancer cachexia. C57BL/6 mice were treated on day 0 with LL2 and, on the same day, either an infusion of CD4⁺CD44^{v.low} cells (\square , $n = 5$), CD4⁺ cells depleted of CD44^{v.low} cells (\blacksquare , $n = 3$), or no CD4⁺ cells (\blacksquare , $n = 4$). In a separate experiment, C57BL/6 mice were treated on day 0 with LL2, and then on days 24 and 25 with either CD4⁺CD44^{v.low} cells (\square , $n = 4$) or no CD4⁺ cells (\blacksquare , $n = 4$). Mice in both experiments were sacrificed on day 28, and skeletal muscle was weighed. The data for each experiment are shown as mean \pm SEM. The level of statistical significance is indicated as * for $p = 0.05$ – 0.01 .

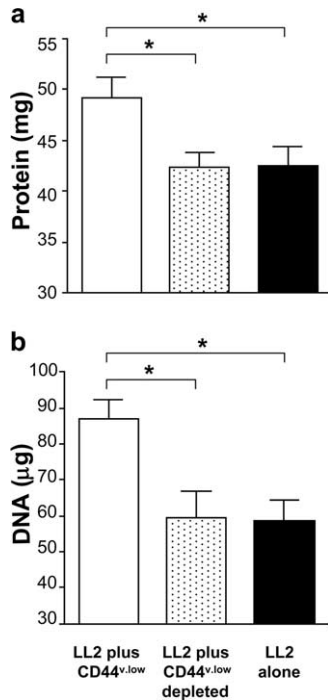


FIGURE 7. CD4⁺CD44^{v.low} cells inhibit skeletal muscle protein and DNA loss in mice with cancer. C57BL/6 mice were treated on day 0 with LL2 and, on the same day, either an infusion of CD4⁺CD44^{v.low} cells (□, *n* = 4), CD4⁺ cells depleted of CD44^{v.low} cells (▤, *n* = 4), or no CD4⁺ cells (■, *n* = 4). Mice from each group were sacrificed on days 25 (*n* = 2) and 27 (*n* = 2) post-LL2 injection and the skeletal muscle was isolated, and the total amount of soluble protein (a) and DNA (b) in each muscle was determined. The data are shown as mean ± SEM, pooled from both time points, and representative of two separate experiments. The level of statistical significance is indicated as * for *p* = 0.05–0.01.

cachexia was clearly evident. Again, the severity of muscle atrophy was significantly reduced in LL2-treated mice that received the CD4⁺CD44^{v.low} cell infusion compared with mice that did not (Fig. 6).

CD4⁺CD44^{v.low} cells inhibit skeletal muscle protein and DNA loss in mice with cancer

Muscle atrophy in cachexia is associated with a dramatic loss of muscle protein. In addition, the cancer cachexia model used in this

study is also associated with a significant loss in muscle DNA (28–30). To determine whether protection from muscle atrophy by infused CD4⁺CD44^{v.low} cells resulted in protection from protein and DNA loss, C57BL/6 mice were injected with LL2 and, on the same day, infused with either CD4⁺CD44^{v.low} cells, CD4⁺ cells depleted of CD44^{v.low} cells, or no cells. Skeletal muscle taken from mice treated with CD4⁺CD44^{v.low} cells 28 days after LL2 injection contained significantly more soluble protein (Fig. 7a) and DNA (Fig. 7b) than skeletal muscle taken from mice treated with either CD4⁺ cells depleted of CD44^{v.low} cells, or no cells.

CD4⁺CD44^{v.low} cell-mediated protection from cachexia is associated with protection from CD4⁺ T cell lymphopenia

C57BL/6 mice injected with LL2 and infused with either CD4⁺CD44^{v.low} cells, CD4⁺ cells depleted of CD44^{v.low} cells, or no cells were sacrificed 28 days later, and lymph nodes were removed. LL2-treated mice that were infused with CD4⁺CD44^{v.low} cells, but not CD4⁺ cells depleted of CD44^{v.low} cells, had significantly greater numbers of CD4⁺CD44^{v.low} (Fig. 8a), CD4⁺CD44^{int} (Fig. 8b), and CD4⁺CD44^{high} (Fig. 8c) cells compared with mice that did not receive a CD4⁺ T cell infusion. The effect of CD4⁺CD44^{v.low} cell infusion on CD4⁺ T cell lymphopenia is similar in the spleen to that seen for lymph node (data not shown). However, data are shown for lymph node rather than spleen because lymphopenia in the lymph node is more dramatic than that seen in the spleen (Fig. 5b). The data show that treatment with CD4⁺CD44^{v.low} cells can inhibit cancer-induced CD4⁺ T cell lymphopenia, and are consistent with the hypothesis that the mechanism that CD4⁺CD44^{v.low} cells use to modulate cachexia involves inhibition of CD4⁺ T cell lymphopenia.

Discussion

Cachexia (1–5) is the term used to describe the overall severe weight loss, muscle wasting, and anorexia seen in patients with a variety of primary disorders, including cancer (32) and AIDS (1); certain autoimmune conditions, including T1D (5), chronic infection (33), and sepsis (34); and is often present in aging individuals with failure to thrive syndrome (2–4, 7–8). Muscle wasting specifically refers to the loss of muscle mass, preferentially in skeletal muscle. It has important clinical consequences, including impaired rehabilitation and shortness of breath (34). Although a number of treatments have been used with some success (35–37), further improvements in therapeutic approaches are needed. In this study, we

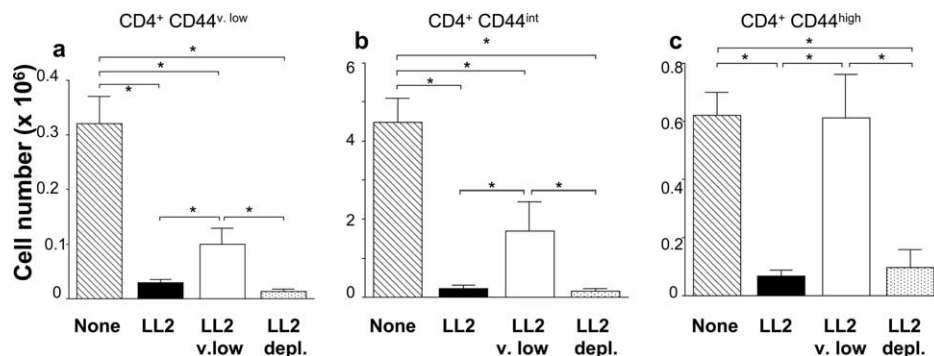


FIGURE 8. CD4⁺CD44^{v.low} cell-mediated protection from cachexia is associated with protection from CD4⁺ T cell lymphopenia. On days 27 (*n* = 2) and 28 (*n* = 2) post-LL2 injection, lymph nodes were isolated from C57BL/6 mice that were treated on day 0 with LL2 and, on the same day, either an infusion of CD4⁺CD44^{v.low} cells (□), CD4⁺ cells depleted of CD44^{v.low} cells (▤), or no CD4⁺ cells (■). ▨, Represent age- and sex-matched untreated mice. The number of CD4⁺ T cells that expressed CD44 at a very low (CD4⁺CD44^{v.low}; a), intermediate (CD4⁺CD44^{int}; b), and high (CD4⁺CD44^{high}; c) density was determined. The data are shown as mean ± SEM, and are pooled from both time points. The level of statistical significance is indicated as * for *p* = 0.05–0.01.

focus on the role of CD4⁺ T cells in the control of muscle wasting in animal models of T1D and cancer cachexia.

Cachexia is often associated with lymphopenia, and cachexia in the two primary disease models used in this study is no exception. The presence of lymphopenia in patients with cachexia is associated with decreased responsiveness to therapy and poor prognosis. Whether lymphopenia is a cause or an effect of cachexia is not yet known. Under healthy conditions, a balance between naive and memory T cell numbers (38–40) and the size of the T cell pool (41–43) are maintained at a constant level by homeostatic mechanisms. We have previously shown that lymphopenia in T1D is associated with a preferential loss of CD4⁺CD44^{v.low} cells, but not CD4⁺CD44^{low} and CD4⁺CD44^{high} cells, at the onset of cachexia (16). Based on these data, we hypothesized that CD4⁺CD44^{v.low} cells promote protection from cachexia and that cachexia ensues, at least in part, as a result of their loss. This hypothesis predicts that infusion of CD4⁺CD44^{v.low} cells into cachexic or precachexic mice might protect from cachexia. Our data show that the transfer of highly purified CD4⁺CD44^{v.low} cells into prediabetic NOD mice significantly inhibits total body weight loss and skeletal muscle atrophy. In contrast, these cells have no effect on the rate of onset and incidence of T1D. Moreover, modulation of cachexia was also seen in mice that were infused with CD4⁺CD44^{v.low} cells, but not CD4⁺ cells that are depleted of CD44^{v.low} cells, after the onset of diabetes. These data suggest that CD4⁺CD44^{v.low} cells protect from cachexia, but not autoimmune diabetes. However, treatment of T1D patients with insulin can inhibit muscle protein breakdown (44, 45) by inhibiting proteolysis and ubiquitin-mediated proteasomal activity (46). Therefore, it was possible that CD4⁺CD44^{v.low} cells inhibited the autoimmune destruction of insulin-secreting β cells to an extent that was insufficient to prevent T1D, but sufficient to protect from cachexia. Analysis of the pancreata from diabetic treated and untreated mice failed to show a significant effect of CD4⁺CD44^{v.low} cells on insulin-secreting β cell mass in diabetic NOD mice. These data strongly suggest that these CD4⁺CD44^{v.low} cells modulate cachexia by a mechanism that is independent of an effect on autoimmune diabetes. Moreover, CD4⁺CD44^{v.low} cells also inhibit wasting and muscle atrophy in a model of cancer cachexia in a nonautoimmune-susceptible mouse strain, further supporting the conclusion that these cells protect from cachexia by a mechanism that is independent of an effect on autoimmunity and insulin secretion. Taken as a whole, the data also suggest that the pathways that lead to cachexia in multiple primary disease states are, at least in part, overlapping. This is consistent with findings that show a common program of changes in gene expression in atrophied skeletal muscle isolated from rats with cancer cachexia and rats with chemically induced diabetes (47). It is important to note that these data do not confirm that the loss of this cell subset is the cause of cachexia in T1D and cancer. However, the data do show that CD4⁺CD44^{v.low} cells are able to protect from cachexia when infused into cachexia or precachexic mice.

Lymphopenia can lead to organ-specific autoimmunity (48, 49), and a resolution of the lymphopenia can result in a resolution of autoimmune pathology (50). However, because skeletal muscle has not been described as a target for autoimmunity, it is unlikely that modulation of cachexia by CD4⁺CD44^{v.low} cells involves mechanisms that are relevant to protection from autoimmunity that is specific for skeletal muscle. Additional support for the conclusion that the mechanism of protection exerted by CD4⁺CD44^{v.low} cells on cachexia does not involve protection from autoimmunity comes from the analysis of the phenotype of the CD4⁺CD44^{v.low} cell population (16). Thus, CD4⁺CD44^{v.low} cells do not express CD25 (51–53), CD38 (54), and CD45RB^{low} (55, 56), cell surface markers that

define regulatory cell subsets known to inhibit autoimmunity. In addition, the regulatory cell marker Foxp3 (57, 58) is also not expressed by CD4⁺CD44^{v.low} cells (J. Davies, unpublished observation). Naive CD4⁺ T cells that do not express regulatory cell markers have been shown to inhibit wasting caused by autoimmune colitis, but not wasting caused by cachexia (50).

CD4⁺ T cells expressing the lowest two densities of CD44 (denoted as CD44^{v.low} and CD44^{int} in this study) are generally considered naive CD4⁺ T cells (CD44^{low}), whereas cells that express a high density of CD44 (CD44^{high}) are generally considered memory CD4⁺ T cells (17, 18). Therefore, based on the density of expression of CD44, CD4⁺CD44^{v.low} cells are naive CD4⁺ T cells. CD4⁺CD44^{v.low} cells also express a high density of CD62L and intermediate/high density of CD45RB (16), further suggesting their naive status (19–21). In addition, whereas activated CD4⁺ T cells express CD25 (59) and CD38 (60), the CD4⁺CD44^{v.low} cell subset expresses neither CD25 nor CD38 (16), suggesting this cell subset is a resting naive CD4⁺ T cell. However, our data do not exclude the possibility that this cell subset is not functional in its naive, unactivated state, and that the CD4⁺CD44^{v.low} T cell subset might only delay cachexia after activation and/or differentiation into a memory cell subset.

In addition to modulating cachexia, CD4⁺CD44^{v.low} cell infusion significantly reduced the extent of CD4⁺ cell lymphopenia in the CD44^{v.low}, CD44^{int}, and the CD44^{high} subsets, protection of CD4⁺CD44^{high} cell numbers being the most significant. Inhibition of lymphopenia by CD4⁺CD44^{v.low} cells might result from differentiation and proliferation to CD4⁺CD44^{int} and CD4⁺CD44^{high} cells, as well as proliferation to maintain the CD4⁺CD44^{v.low} cell population itself. Alternatively, CD4⁺CD44^{v.low} cells might inhibit the depletion of CD4⁺ T cell subsets. These data strengthen the association between lymphopenia and cachexia by showing, for the first time, that protection from lymphopenia is associated with protection from cachexia. It is tempting to speculate that the mechanism used by CD4⁺CD44^{v.low} cells to protect from cachexia involves protection from CD4⁺ T cell lymphopenia, and in particular, protection of the memory cell pool. Alternatively, protection from cachexia might not involve protection from lymphopenia. Nevertheless, the finding that CD4⁺CD44^{v.low} cells protect from lymphopenia might have important clinical implications in improving responsiveness to therapy, irrespective of whether protection from lymphopenia plays a role in protection from cachexia.

The highly consistent balance demonstrated between T cell subsets in healthy individuals might suggest that such control over T cell subset numbers is necessary to maintain the health of the host. In an attempt to repopulate the depleted lymphocyte pool, lymphopenia is generally followed by proliferation of the remaining memory cell pool (61), and proliferation and differentiation of naive CD4⁺ cells to become memory CD4⁺ cells (62, 63) by homeostatic expansion. We suggest that, particularly under conditions in which the cause of lymphopenia is not removed, as is seen for memory CD4⁺ T cell loss at the onset of T1D (16), homeostatic repopulation might not be equivalent for all immune cell subsets. This might be particularly detrimental if memory cells that secrete procachexic cytokines, such as IFN- γ (64, 65), IL-1 (66–68), IL-6 (66–70), TNF- α (71), and TGF- β (72, 73), are preferentially activated and expanded. Alternatively, CD4⁺CD44^{v.low} cells might inhibit cachexia by promoting the expansion of memory cells that secrete IL-4 (74) and IL-10 (75), cytokines known for their anti-cachexic properties. However, whether cytokine manipulation is a viable treatment strategy for cachexia is currently under debate (76–78). It is important to note that T cells are not required for the onset of cachexia, and that cancer cachexia can be induced with

similar kinetics in immunodeficient and congenic immunocompetent BALB/c recipients (67). However, this does not exclude the possibility that, if the immune system is present, it might play a role in enhancing the effects of cachexia. It is also possible that CD4⁺CD44^{v.low} cells can protect from cachexia in immunodeficient recipients by a mechanism that does not involve inhibiting homeostatic expansion of memory cells.

To our knowledge, this is the first report of an immune cell subset that promotes protection from cachexia, and provides a new approach in the search for novel therapeutics for the treatment of this syndrome. Whether the ability of CD4⁺CD44^{v.low} cells to protect from cachexia reflects a novel function for CD4⁺ T cells or whether it reflects an established function that had not previously been linked specifically to CD4⁺ cells and cachexia has yet to be determined. Nevertheless, these findings provide a new insight into understanding the pathways that control the development of cachexia, and suggest a novel role for the immune system in maintaining skeletal muscle integrity.

Disclosures

The authors have no financial conflict of interest.

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