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Therapeutic Time-restricted Feeding Reduces Renal Tumor Bioluminescence in Mice but Fails to Improve Anti-CTLA-4 Efficacy

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Abstract. *Background/Aim: Dietary interventions like time-restricted feeding (TRF) show promising anti-cancer properties. We examined whether therapeutic TRF alone or combined with immunotherapy would diminish renal tumor growth in mice of varying body weights. Materials and Methods: Young (7 week) chow-fed or older (27 week) high-fat diet (HFD)-fed BALB/c mice were orthotopically injected with renal tumor cells expressing luciferase. After tumor establishment, mice were randomized to ad libitum feeding or TRF +/– anti-CTLA-4. Body composition, tumor viability and growth, and immune responses were quantified. Results: TRF alone reduced renal tumor bioluminescence in older HFD-fed, but not young chow-fed mice. In the latter, TRF mitigated tumor-induced loss of lean- and fat-mass. However, TRF did not alter excised renal tumor weights or intratumoral immune responses and failed to improve anti-CTLA-4 outcomes in any mice. Conclusion: Therapeutic TRF exhibits modest anti-cancer properties but fails to improve anti-CTLA-4 immune checkpoint blockade in murine renal cancer.*

There is growing interest in harnessing dietary interventions to combat not only widespread overweight and obesity in the adult population, but also to boost immune function, blunt tumor growth, and improve cancer treatment efficacy (1, 2). Time-restricted feeding (TRF) is a type of intermittent fasting that limits food intake to a set number of hours each day (3). The beneficial effects of TRF are mediated, in part, by attenuating inflammation and oxidative stress, and restoring protective anti-tumor immunity (2). TRF improves chemotherapy-based cancer treatment outcomes without compromising $CD8^+$ T cell anti-tumor immune responses in all murine tumor models examined thus far (4-8). However, the impact of TRF on the balance between pro-tumor immunosuppressive populations (*e.g.*, myeloid-derived suppressor cells [MDSCs]) and anti-tumor immune mechanisms remains unknown.

At this time, no clinical trial or preclinical study has investigated TRF in combination with cancer immunotherapy. Immune checkpoint blockade agents, such as anti-cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4) and anti-programmed death-1 (anti-PD-1), are designed to enhance protective anti-tumor immune mechanisms and are FDA-approved for the treatment of many types of advanced cancers, including kidney cancer (9, 10). Despite demonstrating clinical benefit, typically <50% of patients receiving immunotherapies experience objective, durable responses (11, 12). Although multiple factors contribute to this lack of response (13, 14), evidence is mounting that modifiable lifestyle factors, like diet and elevated adiposity, can impact therapeutic outcomes (2, 15-17). This is particularly important in the context of renal cancer, where obesity is a known risk factor for cancer development (2, 15, 16, 18). Additionally, two important caveats to published data in preclinical cancer modeling are that these studies were limited to investigating chemotherapy and were conducted using young, lean mice that do not reflect the aging, and frequently overweight, population of cancer patients who receive therapy (19).

Although dietary interventions such as TRF may be effective at reducing tumor burden and/or enhancing chemotherapy or immunotherapy outcomes, concerns remain about the possibility for these approaches to exacerbate the

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loss of lean mass in cancer patients who may already be struggling with cachexia and loss of appetite (20). Therefore, additional studies are needed prior to translating these approaches into clinical use.

Here, we sought to evaluate the safety and efficacy of TRF in mice with established renal tumors. We also asked if TRF could improve anti-CTLA-4 outcomes. Our study is the first to test whether TRF can enhance the efficacy of immunotherapy. To mimic the range of cancer patients seen clinically, we used young, chow-fed mice, as well as older, high-fat diet (HFD)-fed mice that were categorized as either normal weight or overweight. Our findings illustrate the potential benefits and drawbacks of translating TRF use into cancer patients, particularly those receiving anti-CTLA-4 checkpoint blockade.

Materials and Methods

Animals and diets. Female BALB/c mice were purchased from the NCI-Frederick colony maintained by Charles River Laboratories (Wilmington, MA, USA) at 7-8 weeks of age. Upon receipt, all mice were acclimated in-house for one week and fed a standard low-fat chow diet (NIH-31; LabDiet, St. Louis, MO, USA). Mice were then randomized to immediate *in vivo* tumor modeling experiments or to 20 weeks of *ad libitum* (AL) high-fat diet (HFD, catalog #12492; Research Diets, New Brunswick, NJ, USA) feeding to generate normal weight (N-WT) and overweight (OVER-WT) mice. These groups were stratified after 20 weeks on HFD by the median pre-tumor body weight (*i.e.*, N-WT ≤27.1 g, mean=24.9 g *versus* OVER-WT >27.1 g, mean=30.8 g). HFD-matched N-WT and OVER-WT mice were then used for *in vivo* tumor modeling experiments. All mice were housed in standard caging under pathogen-free conditions in 12:12 light:dark cycles (dark cycle: 6 PM – 6 AM) at 22˚C (72˚F average). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham, an AAALAC-accredited institution.

Murine in vivo tumor modeling. The Renca cell line (derived from and syngeneic to BALB/c mice) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), engineered to express firefly-luciferase, and cultured as described (21-23). Cells were confirmed negative for mycoplasma, passaged, and used at the same passage number to limit experimental variation. Intra-renal tumor challenges were performed as described (21-23). At day 6 post-tumor challenge, young, chow-fed (n=4-6/group) or HFD-fed N-WT (n=6-8/group) or OVER-WT (n=7-10/group) mice were randomized to AL feeding or TRF, wherein food was withheld during the light cycle (6 AM to 6 PM). To determine if TRF impacted body weight over time, murine body weights were recorded every four days for young, chow-fed mice and pre-tumor and at sacrifice for HFD-fed mice. Young, chow-fed mice were sacrificed at day 22 and HFD-fed mice were sacrificed at day 25 post-tumor challenge. Bioluminescent imaging (BLI) was used to assess tumor burdens over time in live anesthetized mice or excised lungs at sacrifice. BLI of the primary renal tumor was performed by administering 1 mg of luciferin (GoldBio, St. Louis, MO, USA)/mouse and imaging on the IVIS Lumina III Imager (Perkin Elmer, Waltham, MA, USA) within the University of Alabama at Birmingham (UAB) Small Animal Imaging Facility. Primary renal tumors were excised and weighed. Data were generated from n=2 independent experiments.

In vivo immune checkpoint blockade administration. Anti-mouse cytotoxic T lymphocyte antigen-4 (CTLA-4) antibody (clone UC10-4F10-11; BioXCell, Lebanon, NH, USA) was administered intraperitoneally at a dose of 100 μg/mouse on days 10, 13, and 16 or 7, 10, 13, and 16 following tumor challenge where indicated.

Quantitative magnetic resonance (QMR) imaging. Mice were transported to UAB's Small Animal Phenotyping Subcore and imaged by QMR to measure fat and lean mass.

Flow cytometry. Renal tumors were homogenized using a gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany). Homogenized tissue was then enzymatically digested with 5 μg/ml Liberase (Millipore Sigma, St. Louis, MO, USA) and 37.5 μg/ml of DNase I (Millipore Sigma) at 37˚C for 30 min in a shaking incubator, and then passed through a 70 μm filter to yield single-cell suspensions. Cells were counted and stained with Zombie Aqua Fixable Viability Dye (Biolegend, San Diego, CA, USA) followed by TruStain FcX (Biolegend) to block Fc receptors. Cells were then stained with saturating concentrations of conjugated antibodies (Biolegend). Results were obtained from multiparameter flow cytometry using an Attune NxT Flow Cytometer (ThermoFisher Scientific, Waltham, MA, USA) and analyzed with FlowJo software (BD Biosciences, San Jose, CA, USA). The exclusion of doublets was accomplished by FSC-A/FSC-W gating, and dead cells were excluded *via* Zombie Aqua Viability Dye. Boundaries for positive events were objectively determined using fluorescence minus one (FMO) controls.

Statistical analysis. All data were assessed for normality (Shapiro-Wilk normality test) and equal variances, and either parametric or nonparametric analyses were used to detect differences between treatment groups. For studies involving two groups, unpaired Student's *t*-tests or nonparametric Mann-Whitney *U*-tests were performed as appropriate. For three or more groups, one-way ANOVA or nonparametric Kruskal-Wallis tests were performed as appropriate. Pairwise comparisons between groups of interest using Dunn's post hoc tests were performed to correct for multiple comparisons. For experiments examining repeated measures over time between two or more groups, two-way ANOVAs were performed with repeated measures or mixed models design as necessary, followed by post hoc multiple comparison tests (Bonferroni for two groups, Dunnett's for more than two). All statistical analyses were performed using Prism 8 (GraphPad Software; La Jolla, CA, USA). All data are presented as the mean plus or minus the standard error of the mean. Asterisks designate significance using parametric testing ($*p<0.05$, $**p<0.01$, $***p<0.001$); whereas, pound signs designate significance using non-parametric testing ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$). Non-significant trending *p*-values are indicated.

Results

TRF mitigates tumor-induced losses of lean and fat mass but does not diminish renal tumor growth in young chow-fed mice. To evaluate the effects of therapeutic TRF on renal

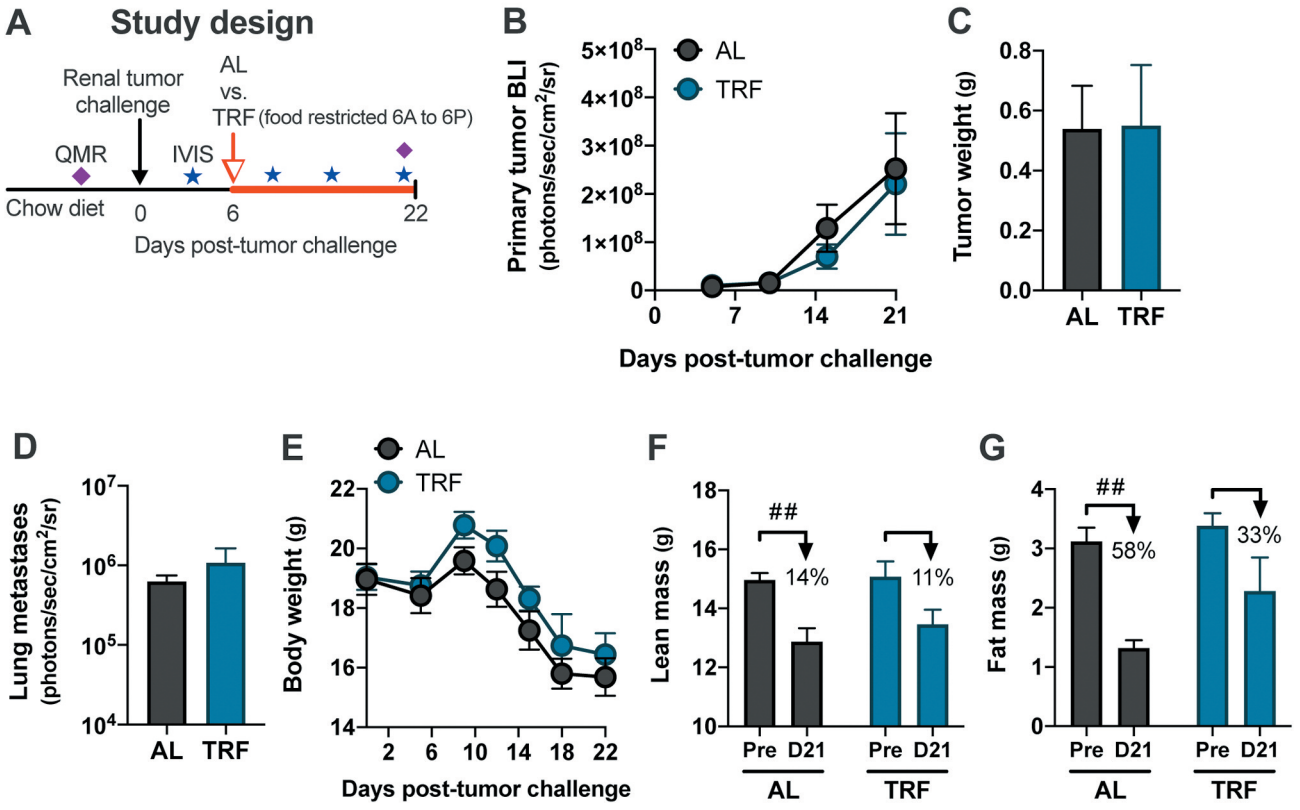

Figure 1. *Time-restricted feeding (TRF) does not alter primary renal tumor outgrowth, spontaneous lung metastases, or body weights in young, chow-fed mice but does mitigate tumor-induced reductions in lean and fat mass. (A) Experimental design for young, chow-fed mice. (B) Primary tumor bioluminescence (BLI) over time (p=0.794), (C) excised renal tumor weights (p=0.662), and (D) spontaneous lung metastases (p=0.931). (E) Body weight (p=0.523) over time. (F) Lean body mass for ad libitum (AL) (p=0.002) and TRF mice (p=0.095) and (G) fat mass for AL (p=0.002) and TRF (p=0.310) mice pre-tumor versus day 21 post-tumor. Data from n=2 independent experiments. ##non-parametric test, p<0.010.*

tumor outgrowth, young chow-fed BALB/c mice were injected intra-renally with the murine renal tumor cell line Renca, which has been engineered to express firefly luciferase (Rena-luciferase). At day 6, mice were randomized to an AL or TRF schedule (Figure 1A; n=5-6/group). No differences between groups were observed for primary tumor outgrowth over time determined by bioluminescence (BLI) imaging (Figure 1B; two-way ANOVA, Group x Time, $F_{(3,11)}$=0.34, p=0.794), excised renal tumor weights (Figure 1C; Mann-Whitney, p=0.662), or spontaneous lung metastases (Figure 1D; Mann-Whitney, p=0.931). TRF had no impact on body weight over time (Figure 1E; two-way ANOVA, Group x Time, $F_{(3,11)}$=0.63, p=0.523). In AL-fed mice, lean body mass (Figure 1F) was significantly reduced at day 21 post-tumor challenge *versus* pre-tumor challenge (14% reduction; Mann-Whitney, p=0.002). TRF blunted the loss of lean mass (11% reduction; Mann-Whitney, p=0.095). Fat mass (Figure 1G) was also significantly reduced at day 21 post-tumor *versus* pre-tumor challenge in AL-fed mice (58% reduction; Mann-Whitney, p=0.002). TRF also blunted this tumor-induced reduction in fat mass (33% reduction; Mann-Whitney, p=0.310). Thus, although TRF did not delay renal tumor outgrowth in young chow-fed mice, it did mitigate deleterious losses of lean and fat mass in these animals.

TRF reduces primary renal tumor bioluminescence without altering excised tumor weights or spontaneous lung metastases in mice on HFD. To ascertain whether TRF had more potent anti-cancer activity in older mice fed a high-fat diet (HFD), BALB/c mice were challenged intra-renally with Renca-luciferase cells after 20 weeks on the diet. At day 6, mice were randomized to an AL or TRF schedule (Figure 2A; n=14-18/group). Primary tumor BLI over time was significantly different between groups (Figure 2B; two-way ANOVA, Group x Time; $F_{(1,60)}$=10.76, p=0.002), with TRF significantly reducing day 24 tumor BLI by 52%. No differences between groups were observed in excised renal tumor weights (Figure 2C; Mann-Whitney, p=0.382) or spontaneous lung metastases (Figure 2D; Mann-Whitney,

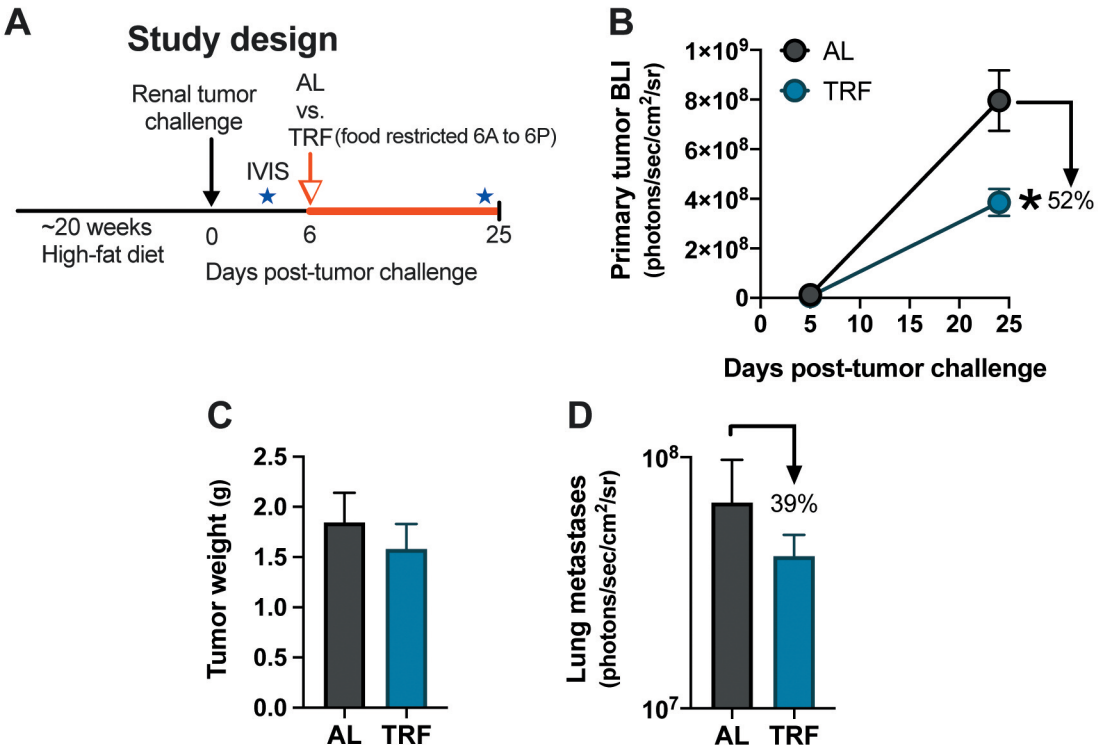

Figure 2. *Time-restricted feeding (TRF) significantly reduces primary renal tumor bioluminescence but does not alter tumor weight at sacrifice in high-fat diet-fed mice. (A) Experimental design for high-fat diet-fed mice. (B) Primary tumor bioluminescence (BLI) over time (p=0.002), (C) excised renal tumor weights (p=0.382), and (D) spontaneous lung metastases (p=0.953). Data from n=2 independent experiments. *parametric test, p<0.050. AL: Ad libitum.*

p=0.953) at day 25 post-tumor challenge. Thus, TRF appeared to reduce tumor cell viability, as evidenced by BLI data, in older mice on HFD.

TRF reduces primary renal tumor bioluminescence in both normal weight and overweight mice on HFD. BALB/c mice on HFD show variable weight gain (24). To determine if varying weight gain altered the response to TRF, after 20 weeks on HFD, mice were stratified by pre-tumor body weight into normal weight (N-WT ≤27.1 g; mean=24.9 g) or overweight (OVER-WT >27.1 g; mean=30.8 g) categories (Figure 3A; Mann-Whitney, p<0.001). Primary tumor BLI over time was significantly different between groups (Figure 3B; n=7-10/group; two-way ANOVA, Group x Time; $F_{(3,11)}$=5.44, p=0.015), with TRF reducing tumor BLI signals in both N-WT (66% reduction, p=0.003) and OVER-WT (44% reduction, p=0.009) groups compared to their respective AL controls. However, excised renal tumor weights were not significantly different between TRF and AL groups, within both N-WT and OVER-WT categories (Figure 3C; Kruskal-Wallis, KW=2.81, p=0.422); post-hoc analysis showed a non-significant but substantial decrease (31% reduction) in N-WT+TRF compared to N-WT+AL. Spontaneous lung metastases were not significantly different between groups (Figure 3D; Kruskal-Wallis, KW=1.17, p=0.759), although N-WT+TRF displayed a non-significant 59% reduction in lung BLI signal compared to N-WT+AL. Thus, TRF appeared to reduce tumor cell viability, as evidenced by BLI data, in both N-WT and OVER-WT mice, and induced a non-significant reduction in tumor weight in N-WT mice.

Tumor-infiltrating immune populations are not altered by TRF in normal weight or overweight mice on HFD. To assess the effects of TRF on immune responses, we analyzed tumor-infiltrating leukocytes ($CD45^+$) at day 25 in N-WT and OVER-WT mice. TRF did not negatively alter the abundance of any specific leukocyte population in N-WT or OVER-WT mice; however, the presence of overweight significantly reduced total viable leukocytes ($CD45^+$) (Figure 4A; n=6-10/group; Kruskal-Wallis, KW=14.40, p=0.002), with both OVER-WT+AL and OVER-WT+TRF groups displaying significant reductions compared to N-WT+AL mice. No significant differences were observed in tumor-infiltrating $CD8^+$ T cells (Figure 4B; Kruskal-Wallis, KW=1.80, p=0.615) or $CD44^+CD8^+$ activated T cells (Figure 4C;

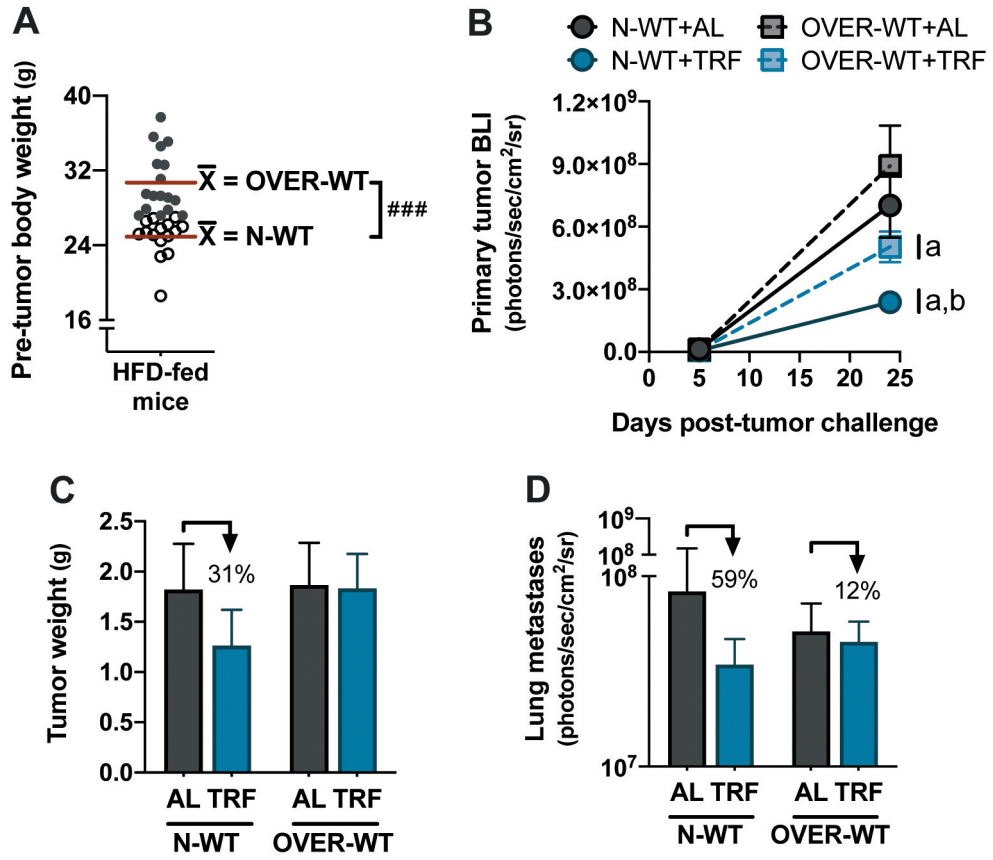

Figure 3. *Time-restricted feeding (TRF) significantly reduces primary renal tumor bioluminescence in high-fat diet-fed normal weight and overweight mice compared to ad libitum (AL) controls but does not alter excised renal tumor weights or spontaneous lung metastases. Experimental design detailed in Figure 2A. (A) Pre-tumor challenge body weight ($\bar{x}$=average body weight, p<0.001 for normal weight; N-WT ≤27.1 g versus overweight; OVER-WT >27.1 g). (B) Primary tumor bioluminescence (BLI) over time (p=0.015), (C) excised renal tumor weights (p=0.422), and (D) spontaneous lung metastases (p=0.759). Data from n=2 independent experiments.* $^{###}$*non-parametric test, p<0.001.* ^{a}*significantly different from N-WT+AL.* ^{b}*significantly different from OVER-WT+AL.*

Kruskal-Wallis, KW=1.37, p=0.713). Total myeloid-derived suppressor cells (MDSCs; $CD11b^{+}CD11c^{-}Ly6G^{+/-}Ly6C^{int//+}$) were significantly different between groups (Figure 4D; one-way ANOVA, $F_{(3,27)}$=4.25, p=0.014), with OVER-WT+AL displaying significant reductions compared to N-WT+AL mice (50% reduction, p=0.023). However, no significant differences were observed in the $CD44^{+}CD8^{+}$ activated T cell to total MDSC ratio (Figure 4E; Kruskal-Wallis, KW=4.57, p=0.207). Thus, TRF did not impair the abundance of tumor-infiltrating anti-tumor immune populations or greatly the ratio of $CD44^{+}CD8^{+}$ activated T cells to MDSCs.

TRF does not enhance anti-CTLA-4 efficacy in young, chow-fed mice with anti-CTLA-4 resistant renal tumors. Because prior studies have demonstrated the efficacy of intermittent fasting or calorie restriction for improving chemotherapy outcomes, we examined whether TRF could improve responses to anti-CTLA-4 in young mice with renal tumors. Young chow-fed BALB/c mice were tumor-challenged, randomized to AL or TRF at day 6, and then further randomized to receive no therapy (NT) or anti-CTLA-4 at days 10, 13, and 16 post-tumor challenge (Figure 5A; n=5-8/group). Under AL conditions, Renca tumors were resistant to anti-CTLA-4 monotherapy, a finding that is consistent with data from previously published studies examining anti-CTLA-4 monotherapy in mice injected subcutaneously with Renca cells (25). No differences between groups were observed in primary tumor BLI over time (Figure 5B; two-way ANOVA, Group x Time, $F_{(9,43)}$=0.15, p=0.998), tumor weight (Figure 5C; Kruskal-Wallis, KW=0.31, p=0.958), or spontaneous lung metastases (Figure 5D; Kruskal-Wallis, KW=0.26, p=0.968). Thus, TRF did not improve anti-CTLA-4 efficacy in young, chow-fed mice with anti-CTLA-4 resistant renal tumors.

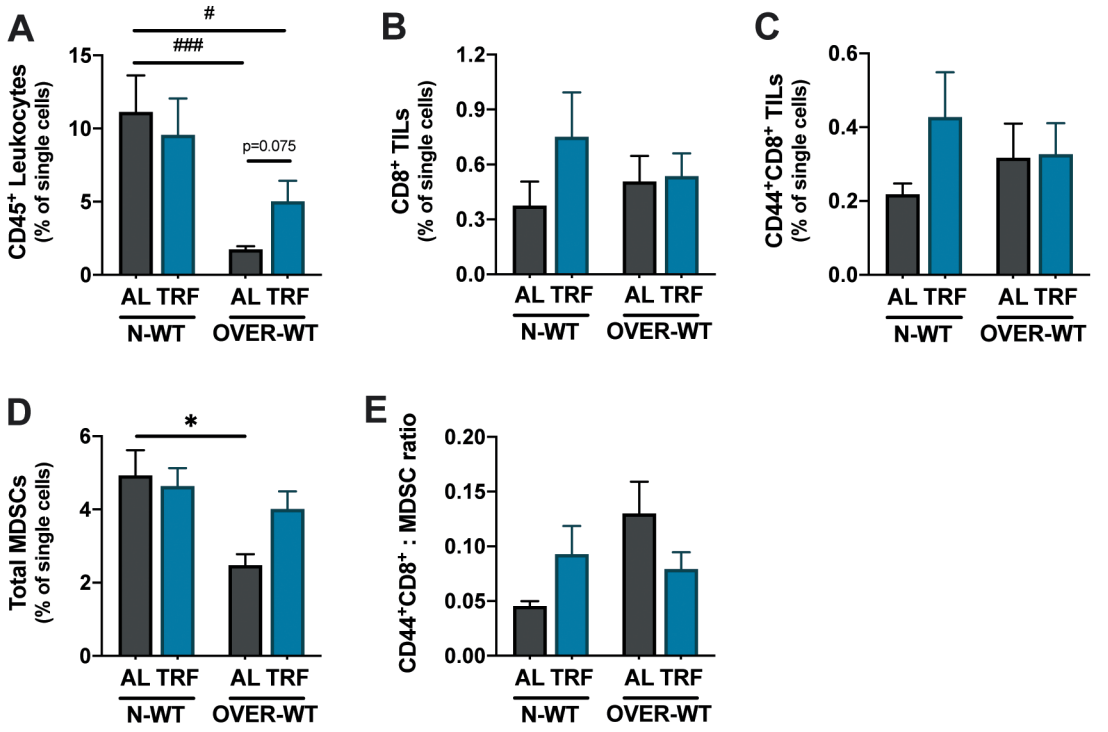

Figure 4. *Time-restricted feeding (TRF) does not alter tumor-infiltrating immune populations in high-fat diet-fed normal weight (N-WT) or overweight (OVER-WT) mice. Experimental design detailed in Figure 2A. Tumor-infiltrating (A) leukocytes ($CD45^+$; p=0.002), (B) $CD8^+$ T cells (p=0.615), (C) $CD44^+CD8^+$ T cells (p=0.713) and (D) total MDSCs ($CD11b^+CD11c^-Ly6G^{+/-}Ly6C^{int/+}$; p=0.014). (E) Tumor-infiltrating $CD44^+CD8^+$ to total MDSC ratio (p=0.207). n=2 independent experiments. Non-parametric test, $^{\#}p<0.050$, $^{\#\#\#}p<0.001$. *parametric test, p<0.050. AL: Ad libitum.*

TRF impedes some of the anti-cancer effects of anti-CTLA-4 in HFD-fed overweight mice with anti-CTLA-4 sensitive renal tumors. We then investigated whether TRF would improve anti-CTLA-4 outcomes in HFD-fed older mice with renal tumors that are sensitive to anti-CTLA-4 monotherapy. BALB/c mice were placed on HFD for 20 weeks to generate N-WT and OVER-WT mice. All mice were tumor-challenged, randomized to AL or TRF, and then further randomized to receive no therapy (NT) or anti-CTLA-4 (n=6-10/group) at days 7, 10, 13, and 16 post-tumor challenge (Figure 6A; n=6-10/group). For the N-WT cohort, primary tumor BLI over time was significantly different between N-WT groups (Figure 6B; two-way ANOVA, Group x Time; $F_{(3,15)}$=3.82, p=0.033), with TRF+NT alone (66% reduction, p=0.001) and TRF+anti-CTLA-4 (54% reduction, p=0.011) groups significantly reducing BLI signals compared to AL+NT mice. Tumor weight was not significantly different between groups (Figure 6C; one-way ANOVA, $F_{(3,25)}$=0.50, p=0.685); however, tumor reductions were observed in TRF+NT (31% reduction, p=0.339) and TRF+anti-CTLA-4 (24% reduction, p=0.454) compared to AL+NT N-WT mice. Spontaneous lung metastases were not significantly different between N-WT groups (Figure 6D; Kruskal-Wallis, KW=0.57 p=0.904); however, lung BLI reductions were observed in AL+anti-CTLA-4 (80% reduction, p=0.116), TRF+NT (59% reduction, p=0.938), and TRF+anti-CTLA-4 (62% reduction, p=0.817) mice compared to AL+NT N-WT mice. For the OVER-WT cohort, primary tumor BLI over time was significantly different between OVER-WT groups (Figure 6E; two-way ANOVA, Group x Time; $F_{(3,13)}$=4.35, p=0.025) with AL+anti-CTLA-4 (53% reduction, p=0.001), TRF+NT alone (44% reduction, p=0.007), and TRF+anti-CTLA-4 (56% reduction, p=0.001) groups having reduced renal tumor BLI signals compared to AL+NT OVER-WT mice. Tumor weights were not significantly different between groups (Figure 6F; Kruskal-Wallis, KW=7.75, p=0.052); however, post-hoc analysis showed a significant reduction in AL+anti-CTLA-4 (31% reduction, p=0.048) compared to AL+NT OVER-WT mice. Spontaneous lung metastases were not significantly different between OVER-WT groups (Figure 6G; Kruskal-Wallis, KW=5.89 p=0.117); however, a trending reduction in lung BLI signal was observed in AL+anti-CTLA-4 (64% reduction; p=0.077) compared to AL+NT OVER-WT mice. Thus, TRF impedes some of the anti-cancer effects of anti-CTLA-4 in mice with anti-CTLA-4 sensitive renal tumors.

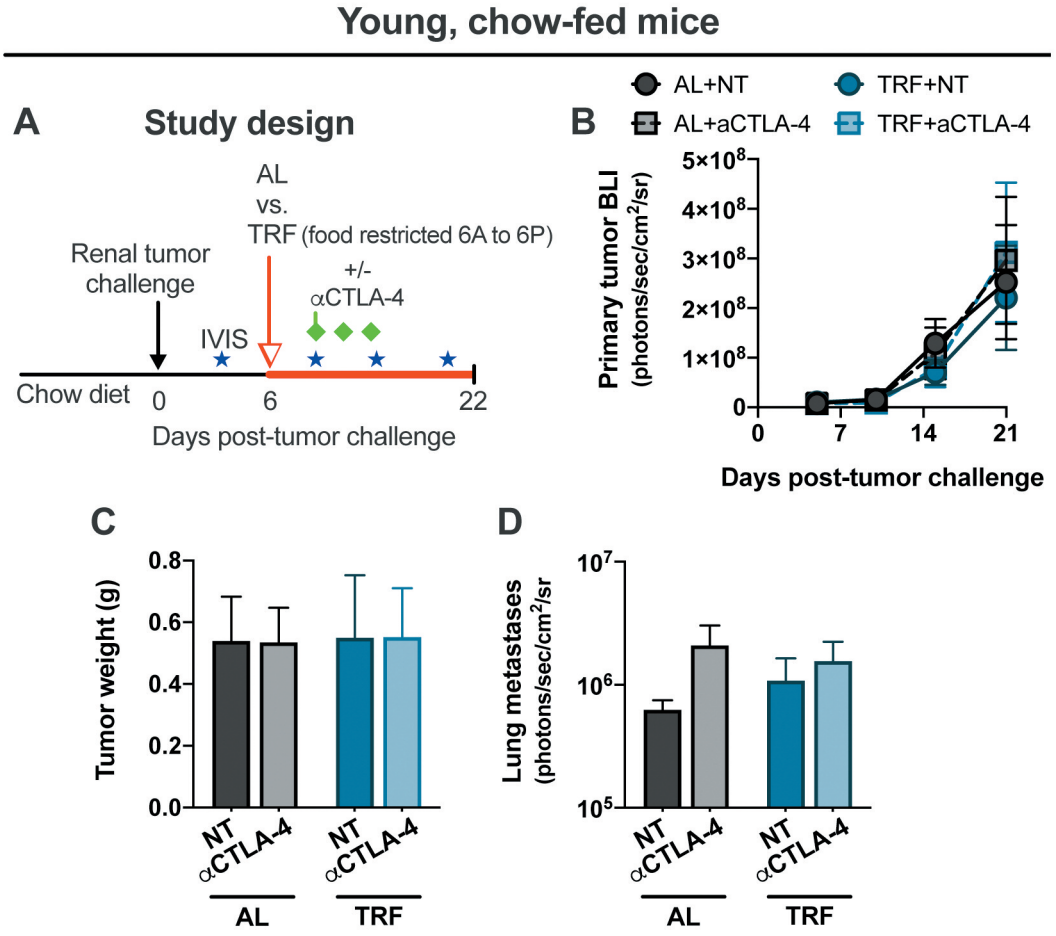

Figure 5. *Time-restricted feeding (TRF) does not enhance anti-CTLA-4 efficacy in young, chow-fed mice. (A) Experimental design for young, chow-fed mice. (B) Primary tumor bioluminescence (BLI) over time (p=0.998), (C) excised renal tumor weights (p=0.958), and (D) spontaneous lung metastases (p=0.968). Data from n=2 independent experiments. AL: Ad libitum.*

Discussion

Here, we examined the effects of therapeutic time-restricted feeding (TRF) alone or in combination with anti-CTLA-4 checkpoint blockade in both normal-weight *versus* overweight and young *versus* old mice with orthotopic renal tumors. In young, chow-fed mice, TRF did not diminish primary tumor bioluminescence (BLI) over time, excised renal tumor weights, or spontaneous lung metastases. However, TRF mitigated cancer-induced cachexia. In older high-fat diet (HFD)-fed mice, TRF significantly reduced primary tumor BLI signals but did not decrease tumor weights or spontaneous lung metastases compared to AL-fed mice. Upon stratifying mice by pre-tumor challenge body weight, we found that TRF significantly reduced primary tumor BLI signal in both normal weight (N-WT) and overweight (OVER-WT) mice compared to AL counterparts and induced a trending reduction in tumor weights and spontaneous lung metastases in N-WT mice. Young, chow-fed mice with renal tumors were highly resistant to anti-CTLA-4 monotherapy, and in this murine model, TRF did not alter anti-CTLA-4 efficacy. HFD-fed N-WT mice were also resistant to anti-CTLA-4 monotherapy; however, TRF induced non-significant reductions in tumor weight and spontaneous lung metastases. In contrast, anti-CTLA-4 monotherapy significantly reduced primary tumor BLI signals and tumor weights, and induced a trending reduction in spontaneous lung metastasis in OVER-WT+AL mice, illustrating enhanced sensitivity to this therapy. Of concern, in these therapy-sensitive mice, TRF negated the beneficial effects of anti-CTLA-4 therapy and renal tumors grew unchecked (*i.e.*, tumor weights from AL+anti-CTLA-4 (0.92 g) were significantly reduced (p=0.015) compared to TRF+anti-CTLA-4 (1.97 g). Thus, our results demonstrate that TRF can produce context-dependent, divergent effects on renal tumor growth, with outcomes influenced by a

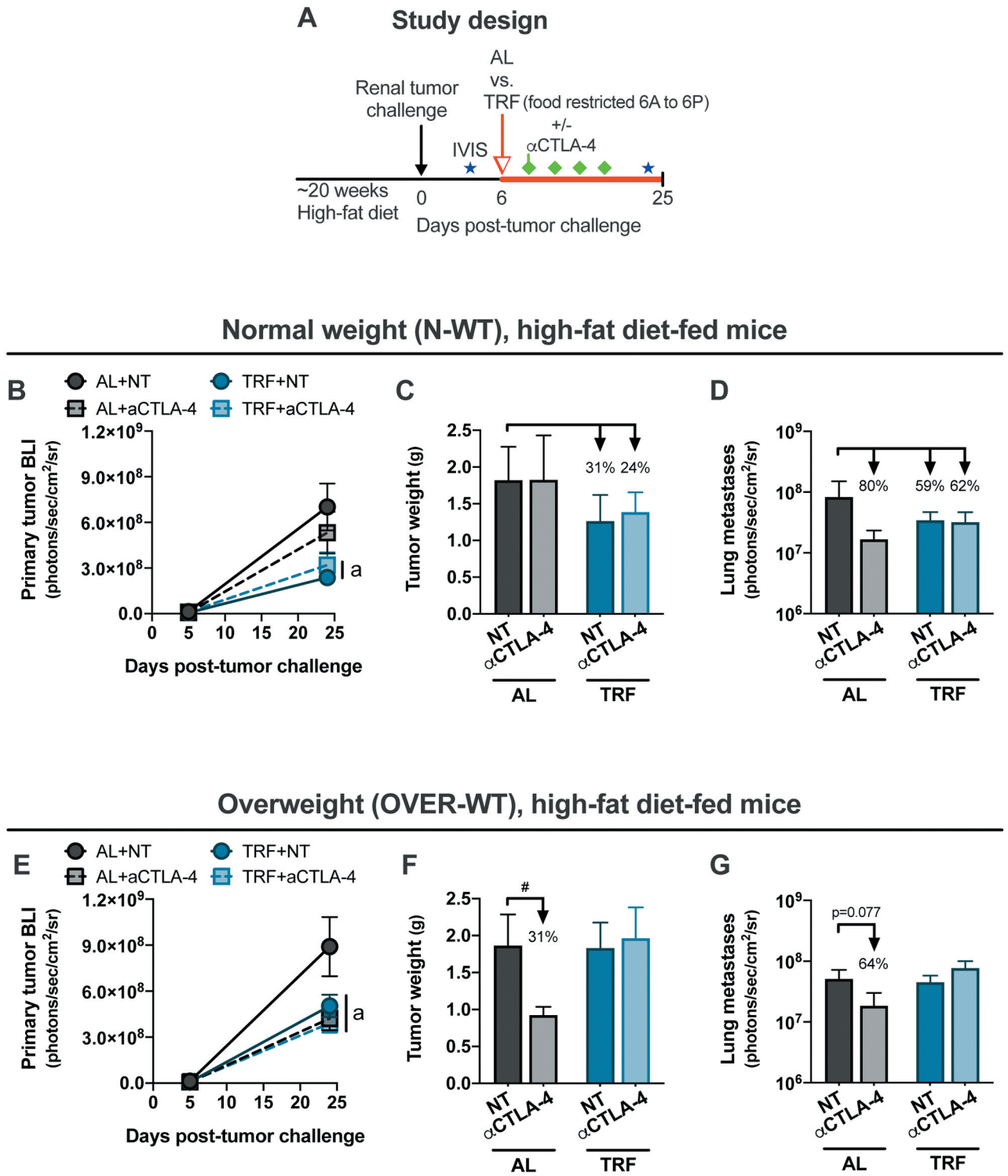

Figure 6. *Anti-CTLA-4 significantly reduces excised tumor weights in ad libitum (AL)-fed, overweight mice but efficacy is not improved by time-restricted feeding (TRF) in high-fat diet-fed normal weight or overweight mice. (A) Experimental design for high-fat diet-fed mice. For the N-WT cohort, (B) primary tumor bioluminescence (BLI) over time (p=0.033), (C) excised renal tumor weights (p=0.685), and (D) spontaneous lung metastases (p=0.904). For the OVER-WT cohort, (E) primary tumor BLI over time (p=0.025), (F) excised renal tumor weights (p=0.052), and (G) spontaneous lung metastases (p=0.117). Data from n=2 independent experiments. [#]non-parametric test, p<0.050. [a]significantly different from AL+NT.*

variety of factors including animal age, diet composition, body weight, and therapy administration. Additional studies are needed before this approach is translated into use in cancer patients, particularly those with renal cancer receiving immune checkpoint blockade.

Chronic calorie restriction reduces tumor growth and enhance anti-tumor immunity in both chow-fed and HFD-fed mice (26, 27). However, chronic calorie restriction in humans is difficult to maintain, and when implemented in murine models, it often results in prolonged periods of fasting, followed by immediate consumption once the daily allotment of food is made available (28, 29). Alternatives to chronic calorie restriction include intermittent fasting strategies, such as short-term fasting, fasting-mimicking diets, and TRF (2, 3). Data from several studies demonstrate that intermittent fasting strategies in both chow-fed and HFD-fed mice can also reduce tumor incidence and growth rates in spontaneously-arising tumor models and can delay tumor growth in transplanted models (8, 20, 26, 27, 30-32). Notably, the majority of these studies administered the dietary strategy prophylactically or used a severe 48-h short-term starvation within the therapeutic window; whereas, in our experiments, TRF was implemented post-tumor challenge.

Tumor progression in the orthotopic Renca model is accompanied by tumor-induced weight loss (Figure 1E). Importantly, TRF did not exacerbate this weight loss in chow-fed or HFD-fed mice. In fact, TRF blunted the tumor-induced weight loss in young, chow-fed mice, preserving both lean and fat mass. This finding is encouraging because it contrasts with prior studies on the use of a short-term 48-h fast in mice with 4T1 tumors, which resulted in a loss of nearly 20% of animal body weight (6). In humans, cancer-associated cachexia is associated with poor overall quality of life, reduced response to cancer therapy, and increased risk of death (33). Pharmacological strategies are attempting to combat cancer-associated cachexia (34), and our data suggest that TRF may be a complementary avenue worth pursuing as a means of preserving lean mass in cancer patients. However, more research is needed to determine if TRF can be safely used to preserve lean and fat mass in cancer patients.

Feeding mice a HFD AL can increase tumor progression by directly promoting tumor growth and by indirectly shifting the immune response from anticancer to pro-cancer (35-39). A caveat to data generated in many HFD-feeding studies is that it is difficult to decipher between the effects of varying diet composition *versus* HFD-induced obesity when reference groups are typically chow-fed, lean controls (15). BALB/c mice represent a mouse strain more resistant to developing diet-induced obesity (40, 41), and we have leveraged this phenomenon to match energy intake and diet composition while comparing outcomes of interest in cohorts of normal weight *versus* overweight mice (42). Here, using HFD-fed BALB/c mice, we were able to broadly assess AL *versus* TRF in HFD-fed mice of varying body weights. TRF significantly reduced primary tumor BLI signals in both N-WT and OVER-WT compared to AL-fed mice. TRF could be reducing nutrient availability to the tumors, reducing the concentration of circulating growth factors (*e.g.*, Insulin-like growth-factor-1), or inducing cell stress and autophagy (43), thereby reducing viable tumor cells as measured by primary tumor BLI signal. It is possible that this beneficial reduction in primary tumor BLI signals may have failed to translate into a reduction in primary tumor weights because of the short intervention window and aggressiveness of the Renca tumor model. Notably, TRF did induce a non-significant but potentially clinically meaningful decrease in spontaneous lung metastasis in HFD-fed mice, a finding that warrants additional research since the majority of cancer deaths are attributable to metastatic disease (44, 45).

The efficacy of many cancer therapies – including chemotherapy, radiotherapy, targeted therapy, and immunotherapy – are mediated in part by functional anti-tumor immune responses or the ability to reinvigorate a functional anti-tumor immune response (46). Interventions that reduce dietary intake have the potential to directly inhibit tumor cell proliferation by reducing nutrient availability, like glucose, to tumor cells (2, 47). However, anti-tumor immune cells, including effector $CD8^+$ T cells, also rely on glucose to clonally expand and support effector mechanisms like cytolytic activity and cytokine secretion (48). Data from previous studies suggest that fasting-mimicking diets and intermittent fasting strategies can actually increase the abundance of effector $CD8^+$ T cells, resulting in enhanced outcomes using anthracycline-based chemotherapy (5, 6). Therefore, we characterized both anti- and pro-tumor immune compartments in treatment naive AL and TRF cohorts to determine whether TRF was blunting protective immune populations. OVER-WT+AL mice displayed a reduction in $CD45^+$ tumor-infiltrating leukocytes compared to N-WT+AL mice. TRF induced a trending increase in the percentage of $CD45^+$ leukocytes in OVER-WT mice. However, the percentages of $CD8^+$ T cells and activated ($CD44^+$) $CD8^+$ T cells were similar within N-WT and OVER-WT groups regardless of feeding strategy. Although OVER-WT+AL mice displayed a reduction in myeloid-derived suppressor cells (MDSC); this failed to translate into an improved activated $CD8^+$ T cell to MDSC ratio (Figure 4G). Thus, in contrast to prior reports on fasting-based dietary interventions, we found that therapeutic TRF did not significantly improve the T cell response to tumors.

In the current study, differential responses to anti-CTLA-4 were observed in N-WT and OVER-WT cohorts. Anti-CTLA-4 monotherapy significantly reduced primary tumor BLI signals and tumor weights, and induced a trending reduction in spontaneous lung metastasis in OVER-WT+AL but not N-

WT+AL mice. This finding mirrors data from a preclinical study where elevated adiposity and increased leptin correlated with enhanced outcomes following anti-PD-1 immune checkpoint blockade in mice with subcutaneous B16-FO melanoma tumors (49). Obesity-induced systemic inflammation could increase immune aging and upregulate inhibitory checkpoint pathways in effector CD8 T cells, providing the necessary targets for immune checkpoint blockade to promote and/or reinvigorate an anti-tumor immune response. The impact of increased adiposity and immunotherapeutic efficacy is being explored clinically (49-52); however, additional research is needed to investigate the effects of obesity-induced dysregulation on anti-tumor immune mechanisms across multiple tumor models and immunotherapeutic strategies to determine if there is a context in which this dysregulation is beneficial to therapeutic efficacy.

The current study contains several limitations. In young, chow-fed mice, TRF alone or in combination with anti-CTLA-4 did not diminish primary tumor BLI over time, tumor weight at sacrifice, or spontaneous lung metastases. Young, chow-fed mice represent a phenotype characterized by low systemic inflammatory and metabolic dysregulation and therefore may offer minimal targets by which TRF can mediate beneficial changes. Additionally, Renca cells injected orthotopically represents a rapid and aggressive tumor model. The limited time period in which TRF was administered in our current study to both young, chow-fed and older, HFD-fed mice may have been insufficient to normalize over-eating-induced perturbations in inflammatory and metabolic mediators. Also, two-fold improvements were observed for multiple experimental endpoints, yet due to limited statistical power and inter-animal variability, these findings failed to reach statistical significance and would likely benefit from increasing sample numbers per groups. Future studies are needed to investigate both a prophylactic TRF administration in Renca tumor-bearing animals, and TRF initiated post-tumor resection to investigate additional applications of this intervention strategy.

Overall, our current study suggests that TRF alone has modest anti-cancer effects that vary by the experimental conditions. Our study is also the first to test whether TRF can act in combination with immune checkpoint blockade to reduce tumor growth, with our current data suggesting that it does not improve the efficacy of anti-CTLA-4 monotherapy in aggressive renal cancer. It is imperative to understand the interactions between overnutrition and immune responses alone and following immunotherapy administration, as well as to determine whether novel strategies, like TRF, can reverse the negative effects of overnutrition to impair tumor growth and/or enhance immunotherapy responses. Identifying molecular and cellular mediators that link overnutrition and the immune response to tumors, with or without immunotherapy administration, and then determining if TRF can alter these mediators to improve outcomes will provide a strong rationale for translating positive findings into clinical applications.

Conflicts of Interest

The Authors have declared that no conflicts of interest exist in relation to this study.

Authors' Contributions

WJT, CP, and LN participated in the conception and design of the work. WJT, RO, JTG and LN performed experiments, data acquisition, and analysis. WJT, RO, JTG, CP, and LN participated in data interpretation, manuscript writing and revision, and approval of the final manuscript for submission and publishing. All Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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