Dietary Protein Restriction Reprograms Tumor-Associated Macrophages and Enhances Immunotherapy

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Abstract

Purpose: Diet and healthy weight are established means of reducing cancer incidence and mortality. However, the impact of diet modifications on the tumor microenvironment and antitumor immunity is not well defined. Immunosuppressive tumor-associated macrophages (TAMs) are associated with poor clinical outcomes and are potentially modifiable through dietary interventions. We tested the hypothesis that dietary protein restriction modifies macrophage function toward antitumor phenotypes.

Experimental Design: Macrophage functional status under different tissue culture conditions and in vivo was assessed by Western blot, immunofluorescence, qRT-PCR, and cytokine array analyses. Tumor growth in the context of protein or amino acid (AA) restriction and immunotherapy, namely, a survivin peptide-based vaccine or a PD-1 inhibitor, was examined in animal models of prostate (RP-B6Myc) and renal (RENCA) cell carcinoma. All tests were two-sided.

Results: Protein or AA-restricted macrophages exhibited enhanced tumoricidal, proinflammatory phenotypes, and in two syngeneic tumor models, protein or AA-restricted diets elicited reduced TAM infiltration, tumor growth, and increased response to immunotherapies. Further, we identified a distinct molecular mechanism by which AA-restriction reprograms macrophage function via a ROS/mTOR-centric cascade.

Conclusions: Dietary protein restriction alters TAM activity and enhances the tumoricidal capacity of this critical innate immune cell type, providing the rationale for clinical testing of this supportive tool in patients receiving cancer immunotherapies. Clin Cancer Res; 24(24); 6383–95. ©2018 AACR.

Introduction

Epidemiologic studies have linked diet to cancer incidence and mortality (1–3). Although no single dietary factor is the sole contributor to cancer, high consumption of animal fat (4), dairy products (5), and red meat have been linked to increased incidence and cancer-related mortality (3). These foods also contribute to higher levels of circulating insulin-like growth factor (IGF)-1, a potent mutagen and mTOR pathway driver linked to tumor progression (2). Conversely, high consumption of vegetables, legumes (6), and fish (7) is linked to reduced cancer incidence, progression, and mortality (3). Many of the modifiable risk factors tie into the Akt/mTOR and insulin/IGF-1 axes and impact immune infiltration into the tumor microenvironment (TME; ref. 8).

Macrophage infiltration is pivotal for tumor immune evasion, angiogenesis, growth, and metastasis. Macrophages assume different functional states, reflecting a continuum of immune-activating (i.e., M1-like) to immune-suppressive (i.e., M2-like) phenotypes, largely affected by stromal- or tumor-derived factors released systemically or locally within the TME. The transition from one functional state to another has been termed ‘polarization’ due to the unique plasticity of the same macrophage population to adopt and respond to the inflammatory milieu. Macrophage polarization and function in turn are affected by various signaling pathways and transcription factors. Activation of the nutrient-sensing mTOR pathway drives macrophage polarization toward a protumor M2 subtype, which has been linked to tumor progression and immune-suppressive activity (9–11). Conversely, inhibition of the mTOR pathway results in a shift toward an antitumor M1 subtype (10).
Translational Relevance

Nutrition and cancer represents an exciting field for laboratory and clinical research. However, to date, the role of specific dietary modifications in altering the immune system and contributing to tumor response to immunotherapies remains unclear. In this study, we report that dietary protein/methionine restriction has a significant effect on reprogramming tumor-associated macrophages toward a more tumor-protective phenotype. More importantly, our data suggest that dietary protein restriction enhances the antitumor effects of immunotherapies in two animal models of prostate and renal cell carcinoma. This finding has translational significance as it supports the rationale for dietary protein restriction in cancer patients receiving immunotherapies such as immune-checkpoint inhibitors.

In response to nutrients and growth factors, the mTOR pathway controls pro-/anti-inflammatory cytokine expression in monocyte-derived macrophages and myeloid dendritic cells (11, 12). Direct inhibition of mTOR has an impact on macrophage cytokine production at both the transcriptional and translational levels (13), and in selective induction of death of the M2 subtype, a shift toward an M1 phenotype, and impairment of myeloid-derived suppressor cell (MDSC) development (10, 12).

In this study, we tested the hypothesis that dietary protein/amino acid (AA) restriction redirects macrophage polarization and function toward an antitumor phenotype. Dietary reduction of specific AAs, such as methionine or cysteine, shifted the presence of TAMs from a tumor-promoting toward a more tumor-suppressive phenotype and modulated immune cell function. Overall, this study suggests that dietary modifications may enhance and promote more durable responses to immunotherapies in cancer patients.

Materials and Methods

Cell lines and culture of bone marrow–derived macrophages (BMDMs)

RP-B6-Myc cells generated from the B6-Myc transgenic mouse model (14) were serially cultured in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin. Bone marrow was harvested from C57Bl/6 (H-2b) mice, plated at 1 × 105 cells/mL, cultured in RPMI media containing 1% penicillin/streptomycin, 1.5% L-Hepes (1M stock), 1% L-glutamine (200 mmol/L stock), 1% NEAA (nonessential amino acids; 100× stock), 1% sodium pyruvate (100 mmol/L stock), and 2 µM β-mercaptoethanol, and treated with 30 ng/mL M-CSF (PeproTech or BioLegend). Cells were allowed to adhere and differentiate for 5 days. On day 5, media were changed to either control [10% FBS, L-cystine (270 mmol/L), L-glutamine (300 mg/L), L-methionine (0.1 mmol/L; 15 mg/L)], 1/2 methionine [10% FBS, L-cystine (270 mmol/L), L-glutamine (300 mg/L), L-methionine (0.05 mmol/L; 7.5 mg/L)], or 1/2 methionine 1/3 cysteine [10% FBS, L-cystine (90 mmol/L), L-glutamine (300 mg/L), L-methionine (0.05 mmol/L; 7.5 mg/L)] media. After 24 hours, cells were treated with IFNγ (20 ng/mL; PeproTech) and LPS (100 ng/mL, Sigma-Aldrich) to prime for M1 macrophage differentiation or IL4 (5 ng/mL; PeproTech) for M2 macrophage differentiation.

Immunofluorescence

Tissue specimens were fixed for 24 hours in neutral buffered formalin, paraffin-embedded and sectioned (5 µm) as previously described (15). Tissue sections were deparaffinized and rehydrated through serial graded xylene and alcohol washes. H&E staining was performed using the standard methods. For F4/80 pan-macrophage IHC staining, antigen unmasking was achieved by incubating the slides in proteinase K solution for 20 minutes at 37°C. For IHC staining, sections were further incubated in hydrogen peroxide to reduce endogenous activity. We then blocked the tissues with 1% BSA in phosphate-buffered saline (PBS) and incubated the slides overnight with F4/80 primary antibody (1:1,000, clone BM8, eBioscience 14-4801) at 4°C. After primary antibody incubation, tissue sections were washed, incubated in horseradish-conjugated anti-rat secondary antibody per the manufacturer’s protocol (Vector Laboratories), enzymatically developed in diaminobenzidine (DAB) and counterstained with hematoxylin. Tissue sections were then dehydrated and mounted with coverslips sealed with cytoseal 60 (Thermo Scientific). Isotype negative controls were used for evaluation of specific staining. For immunofluorescence staining (IF) of tissue sections, antigen unmasking was achieved by boiling the sections in 10 mmol/L sodium citrate (pH6) followed by serial washes and blocking in 1% rat serum, 5% FBS in PBS-T (PBS + 0.1% Tween 20), costained with either CD206 (Alexa Fluor 488, clone C0668C2, BioLegend 141709) and F4/80 (Alexa Fluor 594, clone BM8, BioLegend 123140) or MHC II (FITC, I-A/I-E Clone M5/114.15.2, BioLegend 107605) and F4/80 and incubated overnight at 4°C. Post primary incubation, slides were washed and counterstained with either DAPI (4’,6-diamidino-2-phenylindole, dihydrochloride) or Hoechst and mounted with vectashield mounting medium (Vector Laboratories). BMDSMs were fixed with 4% paraformaldehyde and costained for CD206 and F4/80 overnight at 4°C followed by DAPI staining and mounting using vectashield mounting medium. Stained sections were analyzed either under bright field (HIC) or under the appropriate fluorescence wavelength (IF) using a Zeiss Axio microscope or an EVOS FL cell imaging microscope (Life Technologies). Positive cell percentages were determined in a blinded fashion by analyzing four to five random 20× fields per tissue and quantified using ImageJ software.

Flow cytometry analysis

Macrophages, cultured as described above, were washed with ice-cold PBS and exposed to Accutase (BioLegend 423201) for 15 to 20 minutes on ice. Cells were blocked with Fc Block (anti-mouse CD16/32 mAb; BD Bioscience) at 4°C for 15 minutes, and stained with fluorescence-conjugated antibodies against surface markers CD45 (clone 30-F11), CD11b (clone M1/70), F4/80 (clone BM8), MHC II (I-A/I-E; clone M5/114.15.2), and CD206 (MMR; clone C0668C2). Cells were then fixed in DAPI and stained with antibodies against intracellular proteins, including iNOS (clone C2N4) and Arg1 (R&D IC5868A). Antibodies used for staining were purchased from BD Biosciences, BioLegend, eBioscience, and R&D Systems. Stained cells and isotype control–stained cells were analyzed using a FACSAria III (BD) at the Flow Cytometry Core Facility at the Dana-Farber Cancer Institute.
assayed using an LSRII or Fortessa flow cytometer (BD Biosciences). For 
**in vivo** data, splenocytes, tumor cell suspensions, and peripheral blood cells were washed, blocked with Fc Block (anti-mouse CD16/32 mAb; BD Biosciences) at 4°C for 15 minutes, and stained with fluorescence-conjugated antibodies against surface markers. TAM Panel [CD45 (clone 30-F11), CD11b (clone M1/70), F4/80 (clone BM8), MHC II, CD206], MDSC Panel [CD45 (clone 30-F11), CD11b (clone M1/70), Ly6C (clone AL-21), Ly6G (clone 1A8), PD-L1], T Regulatory/T-cell Panel [CD4 (clone RM4-5), CD25, CD8a (clone 53-6.7), and CD3], and PD-1/P-L1 Panel [PD-1, PD-L1, CD45, F4/80, Ly6C, Ly6G, CD11b, CD8, and CD4] antibodies were purchased from BioLegend, eBioscience, or BD Biosciences. Cells were then fixed in permabilization buffer (eBioscience) and stained with Alexa Fluor 488 and 647 secondary antibodies (Bio-Rad) and exposed to chemiluminescence on a BD FACSCanto II cytometer (BD Biosciences). Data analysis was performed using FlowJo (FlowJo LLC, TreeStar), ModFit LT 4.1 software, and GraphPad Prism 7.

**Western blot analysis**

Tumors or BMDMs from each treatment group were lysed using RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitor cocktails (Pierce). Protein concentrations were assessed using a standard BSA assay (Bio-Rad). Protein (50 μg) from each sample was subjected to electrophoresis on 10% SDS-polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membranes. Proteins of interest were detected with the following primary antibodies: CD206 (1:500; Abcam ab64693), pS6 (1:1,000; Cell Signaling 22115), p-eIF2α (1:1,000; Cell Signaling 92715), β-actin (1:1,000; Santa Cruz sc47778), STAT3 (1:1,000; Cell Signaling 49045), pSTAT3 (1:1,000; Cell Signaling 91345), STAT1 (1:1,000; Cell Signaling 91725), pSTAT1 (1:1,000; Cell Signaling 91675), and EZH2 (1:1,000; Cell Signaling 52465) overnight at 4°C. Following incubation with primary antibody, the membranes were probed with an HRP-conjugated secondary antibody (Bio-Rad) and exposed to chemiluminescence per the manufacturer's instructions (Thermo Fisher Scientific) and exposed to film. Quantitative measurements of Western blot analysis were performed with ImageJ and GraphPad Prism 7 software.

**qRT-PCR and microarray analysis**

RNA concentration and purity were determined through measurement of A260/280 ratios with a Synergy H1 Multi-Mode reader. 
DNA was prepared using the iScript kit (Bio-Rad) and qPCR was performed in triplicate for each sample using SYBR Master Mix (Bio-Rad) or the SYBR Select Master Mix (Thermo Fisher Scientific). Samples were run on an Applied Biosystems 7900HT fast real-time PCR system. Sequence Detection Systems software v2.3 was used to identify the cycle threshold (Ct) values and to generate expression curves. Data were normalized to GAPDH expression, and fold change was calculated. The primers used for target genes were: GAPDH 5′-AACACTTGGGCTATTTCTC-3′ and 3′-ACACATTGGAAGATCATCC-5′; NOS-1 5′-ACCCGAGAGTCCTTTCT-3′ and 3′-CCGCACTTGATCCTTCTCAG-5′; Arg1 5′-GGCTGTCCTCAGAGGAACTA-3′ and 3′-ATGAGGAAGCCTTAGCAG-5′; NF-κB 5′-CTTCGCTGAAAGAAGATGCG-3′ and 3′-CCGGTCCAGAGGATCTGAGGAG-3′; Keap1 5′-ATGGCCACACTTCTGTCACAG-3′ and 5′-CTTCGTTGCTAGTGCTCAAGG-3′; ATF4 5′-TCTGATCTGTTGGTTGAAAGTTGA-3′, γIFN 5′-GGCCACCTGTCGACTTTGAATC-3′, CC2 5′-AGGTCCTGTCTCTGTGCTTCG-3′ and 5′-CTGGAGGGTCAGTTTCTGTC-3′; NFAT5 5′-AAGGCAACCTCIAAGGCTGACA-3′ and 5′-TGGAACACACTGTTCCAC-3′. Expressions of the different genes were normalized to GAPDH. Relative expression was calculated using the 2^-ΔΔCt method.

**Proteome profiler**

Tumor tissue was homogenized in PBS containing protease inhibitors. Following homogenization, Triton X-100 was added to a final concentration of 1%, frozen at −80°C, thawed, centrifuged at 10,000 × g for 5 minutes, quantified and assayed according to the manufacturer's protocol. All samples were processed and run on R&D Systems mouse XL cytokine array kit (AOY288). Analyses were performed using HLMImage++ and QuickSpots Tool (Western Vision Software) and GraphPad Prism 7.

**In vivo studies**

RP-B6-Myc cells were injected subcutaneously into male C57BL/6 mice as previously described (16). The experimental diets were prepared and sterilized by irradiation by the Envigo (Harlan Laboratories) facility. A summary of the composition and ingredients of each diet is shown in Supplementary Fig. S1. Tumors grown subcutaneously in C57BL/6 mice were harvested for flow cytometry analysis as described below. All procedures were performed and approved in strict accordance with the Institutional Animal Care and Use Committee at Roswell Park Cancer Institute, Indiana University School of Medicine, and with the NIH Guide for the Care and Use of Laboratory Animal guidelines. Six- to 8-week-old male C57BL/6 mice were ordered from Charles River Laboratories and housed in a sterile, pathogen-free facility. Mice were maintained in a temperature-controlled room under a 12-hour light/dark schedule with water and food ad libitum. For diet and immunotherapy treatment studies, untreated RP-B6-Myc tumors were collected from passing C57BL/6 mice, dissected into ~1 mm³ tumor pieces and implanted subcutaneously into mice. All mice were operated on under sedation with oxygen, isoflurane, and buprenorphine. Upon tumor establishment, ~50 mm³ mice were randomly grouped and placed in either control or treatment groups. Animals were allowed ad libitum access to food and water. Male C57BL/6 mice were randomized, via tumor size using a stratified randomization procedure, into control AIN93G and 80% dietary protein restriction for 8 weeks. Female 5- to 6-week-old Balb/c mice (Charles River Laboratories) were maintained as described above. One week prior to RENCA cell injection, mice were placed into four groups: control diet, control diet + anti-PD-1 (Bio X Cell RPM1-14, rat IgG2a), 7% protein diet, and 7% protein diet + anti-PD-1. Seventy to 80% confluent RENCA-Luc cells were harvested using 0.25% Trypsin (Corning) and suspended in a 1:1 ratio of matrigel (Corning) and HBSS (Gibco), and 10 μL containing 1 × 10⁶ cells was injected under the renal capsule. Mice were imaged via bioluminescent IVIS imaging, and twice weekly measured via body weight.
measurement. All mice were under clinical observation for weight loss, lethargy, and hunched/ruffled appearance. Mice were randomly placed into treatment groups (5–10 mice/group) of diet alone (control vs. 80% methionine restricted or 21% vs. 7% dietary protein) or diet with immunotherapy treatment. Mice in the treatment groups received 20 mg/kg of anti-PD-1 (RP-B6-Myc and RENCA) twice a week and survivin peptide vaccine (RP-B6-Myc) 1 mg/mL for 4 weeks. Cldoridine liposomone treatments were administered every 3 days intraperitoneally (100 μL of 100 mg/mL solution). Tumor size and body weight were assessed and recorded twice per week. For tumors implanted subcutaneously, tumor size for the RP-B6-Myc model was measured in a blinded manner twice a week by caliper measurement of two diameters of the tumor (length and width). Measurements were reported as tumor volume (length × width²)/2 = mm³. Body weights were assessed using a weighing scale and recorded in grams. Tissue and blood were collected under aseptic conditions. Blood (400–600 μL) was collected by eye bleeds (terminal) at the end of the experiment. Serum aliquots were stored at −80°C for further analysis. Tumor tissues were excised, weighed, and processed for flow cytometry analysis, snap-frozen, and stored in −80°C, stored in TRIzol (Life Technologies) for RNA analysis, or fixed in 10% buffered formalin or optimal cutting temperature (OCT) medium for histopathology. Endpoint tumor weights were assessed using a weighing scale and recorded in grams. Facial matter was collected from each mouse, snap-frozen, and stored at −80°C for future analysis.

**Statistical analyses**

Data are presented as the mean ± SEM. For in vitro experiments, no animals were excluded from the analyses. The results of gene analysis and flow cytometry were calculated using both Welch t test and one-way ANOVA analyses. Analysis of the proteomic profiling was accomplished by using multiple t tests and two-way ANOVA analysis for each protein. All tests were two-sided, and P ≤ 0.05 was considered significant. All in vitro experiments were repeated at least three times independently to obtain biological triplicates.

**Results**

**Dietary protein and AA restriction inhibits macrophage polarization toward an M2 phenotype**

We first assessed macrophage infiltration into the TME of SCID mice bearing the LuCap23.1 patient-derived xenograft prostate cancer model, which had been fed 20% or 7% protein-based diets (15). We observed no significant difference in the overall number of TAMs (Supplementary Fig. S2A), but an increase in M1-like TAMs along with a decrease in the M2-like phenotype in those mice fed a 7% diet was observed (Fig. 1A and B). Next, we developed an in vitro model to assess the effect of specific AA alterations on macrophage polarization and function by focusing on methionine restriction because it mirrors the effects of calorie restriction (2) or dietary protein restriction (17). BMDMs following polarization with prototypic M1- (IFNγ + LPS) or M2- (IL4) stimuli were collected and analyzed for changes in viability and hallmark features of M1- (iNOS) or M2- (Arg1) type macrophages (Fig. 1C). Although no reduction in the viability of BMDMs was observed after exposure to AA-restricted conditions (Supplementary Fig. S2B and S2C), a significant increase in iNOS and decrease in Arg1 levels occurred (Fig. 1D and E).

**Dietary protein and AA restriction reverses the immunosuppressive function of M2-type macrophages**

Next, we examined whether the observed alteration in the polarization of BMDMs was indicative of increased tumoral activity. Polarized BMDMs were cocultured with the prostate cancer cell line RP-B6-Myc (16). AA restriction alone had a moderate effect on altering the viability of RP-B6-Myc cells following 72 hours of exposure (Supplementary Fig. S3A). Both M1- and M2-type macrophages became increasingly tumoricidal after coculture under AA-restricted conditions (Supplementary Fig. S3B). Further, M2-type macrophages, which impair CD8⁺ T-cell activity in part by reducing granzyme B expression, lose their suppressive capability when cultured in AA-restricted conditions (Supplementary Fig. S3C). In contrast, T cells, activated with anti-CD3 and anti-CD28 antibodies, do not show loss of cell viability or reduction of granzyme B expression (Supplementary Fig. S3D).

STAT1 and STAT3 transcriptionally regulate the differentiation of monocytes/macrophages into antitumor M1 or M2 protumor subtypes, respectively (18). We reexposed "polarized" AA-restricted M1- or M2-type BMDMs to either STAT1 (IFNγ + LPS) or STAT3 (IL6) activators, and STAT1/3 phosphorylation was analyzed. The M2-type macrophages cultured under restricted conditions were the most altered (data not shown). These cells were unable to recover their capacity to phosphorylate STAT3, but rather showed more inhibition of p-STAT3 than the M1 counterpart. M2-type macrophages also showed an increase in their capacity to phosphorylate STAT1, which supports a more tumoricidal M1-type response.

**AA restriction alters cytokine production by BMDMs**

Then, we analyzed the media from our coculture assay and observed that M1-polarized macrophages displayed a cytokine/chemokine profile consistent with an antitumor/proinflammatory phenotype, including CXCL10, CXCL11, CCL2/MCP-1, CXCL9, CCL3, CCL4, and TNFα (Fig. 2A and B), and a reduction in tumor-promoting cytokines and chemokines including IL6, IL10, IL13, and IL33 (Fig. 2C). M2-polarized macrophages, when cultured in AA-restrictive conditions, showed a decrease in proinflammatory cytokines and chemokines, including IL1ra, IL6, IL23, CXCL1, CCL5, and CCL17 (Fig. 2D and E). Because these cytokine/chemokine profiles have been shown to play a crucial role in antitumor immunity (19), we expanded our analysis to a panel of 84 inflammatory proteins (Fig. 2F). This more comprehensive analysis revealed five key downregulated M2-type macrophage–associated targets, IL1ra, IL10, IL13, IL17, and CXCL1, which overlapped with our proteome profiler (Fig. 2G). We recognize that AA restriction elicits a pleiotropic cytokine response that spans a continuum of alterations due to the complex biologic heterogeneity of such an extrinsic intervention. Thus, given the complexity of the impact of AA restriction on the host, it is likely that the resultant cytokine profile is not all-or-none, but rather a blend of proinflammatory or anti-inflammatory markers.

**Dietary protein and AA restriction inhibits tumor growth in syngeneic mouse models of prostate and kidney cancer**

Next, we tested whether in vivo dietary AA or protein restriction would inhibit tumor growth and enhance the effect of...
immunotherapy. To test this hypothesis, we used two syngeneic mouse models, RP-B6-Myc and RENCA (16), which present a high infiltration of M2-like TAMs (Supplementary Fig. S4A; Fig. 3A–F; ref. 20). In support of the role of macrophages in our experimental models, we observed that dietary protein restriction failed to reduce tumor growth under macrophage-depleted conditions (Supplementary Fig. S5). To examine the impact of dietary methionine/cystine restriction (M/CR), we developed an 80% methionine-restricted diet (80MR; Supplementary Fig. S1). Our experimental diet did not contain cysteine, as the transsulfuration pathway can overcome protein restriction (80MR; Supplementary Fig. S2). To assess the immunomodulatory activity of 80MR, we combined dietary modification with two different forms of immunotherapy. The presence of phenotypically PD-L1+ and immunosuppressive myeloid populations in these tumor models (Fig. 3A–F) suggests the use of an anti–PD-1 strategy. Survivin expression, a tumorigenic protein that promotes tumor vascularization and inhibits apoptosis and is associated with poor prognosis in prostate cancer patients (22), has been previously confirmed in Myc-CaP tumors (16), providing a rationale for combination with a survivin peptide–based vaccine in the RP-B6-Myc tumors (23). Anti–PD-1 treatment allows cytotoxic T-cell proliferation and activation in the TME by release of the inhibitory checkpoint (24).

Figure 1.
Protein/AA restriction inhibits M2-like macrophage tumor infiltration and blunts the expression of M2 signatures. A, top, Representative M1 macrophage staining for F4/80 (red) and MHCII (green) for 21% and 7% protein diet cohorts in the LuCaP23.1 model. Bottom, Representative M2 macrophage staining for F4/80 (red) and CD206 (MIR; green) for 21% and 7% protein diet cohorts in the LuCaP23.1 model. B, left, Blinded quantification of M1 macrophage presence in the TME based on random selection of five fields per tissue. Right, Blinded quantification of M2 macrophage presence in the TME. Bottom right, Western blot analysis showing M2 marker CD206 presence in 21% versus 7% protein diet conditions. C, Schematic of in vitro macrophage stimulation. D, Flow cytometry analysis for M1 (CD45+CD11b+ F4/80+ iNOS+) and M2 (CD45+CD11b+ F4/80+ Arg1+) in AA-restricted BMDMs 48 hours after stimulation. The experiment was repeated at least three times independently. Legend: 1. Control media, 2. 1/2 methionine media, 3. 1/2 methionine 1/3 cystine media (M/CR), we developed an 80% methionine-restricted diet (80MR; Supplementary Fig. S1). Our experimental diet did not contain cysteine, as the transsulfuration pathway can overcome protein restriction (80MR; Supplementary Fig. S2). To assess the immunomodulatory activity of 80MR, we combined dietary modification with two different forms of immunotherapy. The presence of phenotypically PD-L1+ and immunosuppressive myeloid populations in these tumor models (Fig. 3A–F) suggests the use of an anti–PD-1 strategy. Survivin expression, a tumorigenic protein that promotes tumor vascularization and inhibits apoptosis and is associated with poor prognosis in prostate cancer patients (22), has been previously confirmed in Myc-CaP tumors (16), providing a rationale for combination with a survivin peptide–based vaccine in the RP-B6-Myc tumors (23). Anti–PD-1 treatment allows cytotoxic T-cell proliferation and activation in the TME by release of the inhibitory checkpoint (24).
Macrophage tumoricidal function, cytokine release, and gene expression are enhanced in M1- and M2-primed BMDMs following AA restriction. **A**, Heat map displaying the differential expression pattern of 40 mouse cytokines, chemokines, and acute phase proteins from M1-differentiated (IFNγ + LPS-treated) macrophages in 1/2 methionine or M [B] (n = 2) and 1/2M 1/3 cystine or C media [C] (n = 2) compared with M1 macrophages in control media [A] (n = 2). **B**, Tumoricidal cytokines and chemokines which were enhanced with MR in M1-polarized macrophages. **C**, Tumor-supportive chemokines and cytokines which were downregulated following AA restriction in M1 macrophages. **D**, Heat map displaying the differential expression pattern of 40 mouse cytokines, chemokines, and acute phase proteins from M2-differentiated (IL4-treated) macrophages in 1/2M [B] (n = 2) and 1/2M 1/3 C media [C] (n = 2) compared with M2 macrophages in control media [A] (n = 2). **E**, Protumor, M2-associated proteins which were downregulated following AA restriction. **F**, Using an RT² profiler mouse inflammatory cytokine and receptor PCR array from Qiagen, a scatterplot showing the expression of upregulated and downregulated genes was generated (n = 2 per group). Red dots represent upregulated genes, black dots represent unchanged genes, and green dots represent downregulated genes. **G**, List of the top downregulated proteins/genes that were represented in both the ARY006 and the mouse inflammatory cytokine and receptor PCR RT² profiler (PAM-Q01A). Results are presented as the mean ± SE. Statistical significance was determined using Student t test with Welch correction. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

Supplementary Fig. S4B. We also observed significant decreases of PD-L1 F4/80+ and F4/80+ CD206+ Arg1+ macrophages in the 80MR cohort compared with the control diet cohort; alterations in M2-type macrophages were not compounded by IT treatment for the RP-B6-MyC model but were further reduced in the RENCA model (Fig. 5A; Supplementary Fig. S4B). The striking difference observed in the 80MR study, relative to the 7% diet, further supports that cystine/methionine restriction is critical to altering the macrophage subtypes within the TME. We also examined the TME for changes in the infiltration of MDSCs (Fig. 5B) and observed significant decreases in both polymorph nuclear (PMN)- and monocytic (M)-MDSC subsets, which typically support tumor growth and possess the potential to differentiate into TAMs (25, 26). By using a proteome profiler array (Ary028) to assess the production of chemokines and cytokines involved in immune cell infiltration (Table S1), we found increased M1-type proinflammatory proteins, including CXCL11/F-TAC, IL1alpha, IL1beta, IL12p40, M-CSF, and IL17A. In the case of IL17, it has been implicated as proinflammatory cytokine when associated with an M1-type macrophage profile, whereas protumorigenic when associated with an M2-type macrophage profile (27, 28). In addition to an increased M1-type macrophage signature, we observed a concomitant reduction in M2-type and tumor growth/progression-associated proteins, including C-reactive protein, FGF acidic, IL33, leptin, and MMP9 (Fig. 5C).

The TME infiltration of CD8+ CD3+ T cells, a key component of antitumor immunity, has been linked to improved patient prognosis (29). Interestingly, we observed a significant increase in tumor-infiltrating CD8+ CD3+ T cells and cytotoxic granyme B+ CD8+ CD3+ T cells in the 80MR and even further in the 80MR + IT cohorts (Fig. 5D). We also observed a significant increase in circulating CD8+ CD3+ T cells in the 80MR cohorts compared with the controls (Fig. 5E) along with an increase in the ratio of CD8+ CD3+ T cells to M1- or M2-type macrophage, as well as PMN- or M-MDSCs in response to dietary M/CR. Further analysis of the T-cell infiltrates within the TME revealed reduced coexpression of CD8 and TIM-3 (Fig. 5F), providing additional evidence that the TME is transitioning away from a protumor environment (30, 31). We also observed significant increases in the IT groups for the CD8+ CD3+ to MDSC ratio (Supplementary Fig. S6A–S6D; ref. 24).
AA restriction modulates the molecular programming of macrophage polarization

AA restriction and oxidative stress have been shown to upregulate the cystine glutamate transporter, which regulates intracellular reactive oxygen species (ROS) that distinguish M1/M2 macrophage polarization phenotypes, xCT expression (32, 33). We observed that xCT (34) is highly expressed in M1-type macrophages (Supplementary Fig. S7A) and increased in AA-restricted BMDMs, and the mTOR pathway and redox sensitive transcription factor, Nrf2, is downregulated along with phospho-S6 ribosomal protein (pS6; Fig. 6A–C). Similarly, the negative regulator of Nrf2, Keap1, which leads to ROS/mTOR-dependent Nrf2 ubiquitination (35), is significantly upregulated under AA-limited conditions (Supplementary Fig. S7B). Atf4 is a transcription factor for xCT linked to LPS-induced TLR4 signaling and cytokine production (36), and when phosphorylated, eIF2α is unable to bind to and inhibit Atf4 (37). eIF2α phosphorylation was observed under AA-restricted conditions (Fig. 6D), in conjunction with an increase in Atf4 gene expression for M1- and M2-type macrophages (Supplementary Fig. S7C). Increased ROS in macrophages is indicative of M1-type polarization and increased proinflammatory cytokine release (33). There was a significant increase in ROS production in both M1- and M2-type macrophages under the AA-restricted conditions (Fig. 6E), along with Nfat5, which promotes the expression of numerous proinflammatory cytokines, including Ccl2, TNFα, and IL12, and is required for iNOS induction (ref. 38; Supplementary Fig. S7D). Additionally, we observed a significant increase in the production of several proinflammatory cytokines, including Ccl2, validated by quantitative RT-PCR analysis (Fig. 6F; ref. 39).

Dietary M/CR alters the polyamine synthesis pathway in the TME

Inhibition of the polyamine biosynthesis pathway has been shown to alleviate tumor escape, enhance T-cell responsiveness, and inhibit the differentiation and accumulation of immunosuppressive myeloid cells, including M2-type TAMs and MDSCs, while promoting M1-type TAMs (40). We did not observe an overall reduction in polyamines either in vitro (Supplementary Fig. S8A) or in vivo with M/CR, but there was a significant reduction of spermine (SPM) in the TME of our RP-B6-Myc model (Supplementary Fig. S8B). This finding, combined with the increased spermidine (SPD) to SPM and
putrescine (PUT) to SPM ratios (Supplementary Fig. S8C) in the TME, indicates that our intervention may be inhibiting the polyamine biosynthesis pathway (41). To examine whether this may affect macrophage polarization, we next inhibited the synthesis of polyamines, using difluoromethylornithine (DFMO). In our system, the inhibition of polyamine synthesis resulted in an increased M1-type macrophage polarization in control conditions, and a significant reduction of M2-type macrophages in both control and AA-restricted media, suggesting that DFMO is sufficient to phenocopy the effects of AA restriction (Supplementary Fig. S8D), and inhibition of the polyamine biosynthesis pathway may contribute to macrophage polarization in our model. Upstream of polyamine biosynthesis, the SAM (S-adenosylmethionine):SAH (S-adenosylhomocysteine) ratio is directly affected by circulating methionine. Although we saw no significant alteration in the overall levels of SAM and SAH in our B6-Myc tumor model (data not shown), we did find a significant negative correlation (Pearson correlation: \( R = 0.652, P < 0.05 \)) in SAH and SAM levels in the 80MR cohort, with no clear correlation between the SAM and SAH levels in the control diet cohort (Pearson correlation: \( R = 0.184, P > 0.05 \)).

Dietary M/CR alters the gut microbiota, which may contribute to immune system alterations

Gut microbiota is influenced by dietary protein (42) and has been linked to immune activation in response to cancer treatments (43). Bacterial genera, which have been linked to cancer progression, include *Prevotella* and *Ruminococcus*, whereas *Lactobacillus* is negatively correlated (44). *Prevotella* and *Ruminococcus* are increased in colorectal cancer and have detrimental effects on the immune system by altering T-cell and myeloid cell function. Conversely, *Lactobacillus* is known to play an immune-stimulatory role that may involve the TRAIL and IFN-\( \gamma \) pathways (45, 46). The microbiomes of mouse fecal samples from the control and 80MR groups, determined by small bacterial ribosomal subunit (16S) sequencing, were clearly distinguished by Venn diagram and principal coordinate analyses of operational taxonomic units (OTU; Supplementary Fig. S8D). Next, we investigated OTUs, which revealed dramatic shifts in the microbial community of the 80MR relative to the control diet cohort. Consistent with our community-wide beta diversity and OTU profiling, we observed changes in numerous bacterial populations and focused on *Ruminococcaceae*, *Prevotellaceae*, and *Lactobacillaceae*, which have been directly linked to cancer progression and immune system alterations.

Figure 4.
Translation of *in vitro* M/CR into the *in vivo* RP-B6-Myc and RENCA models results in significant inhibition of tumor growth and enhances IT responses. Prostate cancer (CaP) model, RP-B6 Myc: A, top, treatment schema. Two weeks following RP-B6 Myc tumor implantation, mice were begun on a treatment regimen of twice-weekly anti-PD-1 (20 mg/kg) and weekly survivin peptide vaccine (1 mg/mL). Graph, Tumor volume measured as mm\(^3\) (n = 10 mice/group). Comparison of the control + IT and 80MR + IT tumor growth curves. B, The total tumor burden (grams) at day 32 compared with the control AIN93G. C, top, treatment schema. Graph, Tumor volume measured as mm\(^3\) (n = 10 mice/group). Renal cell carcinoma model, RENCA. D, top, treatment schema. Graph, the total RENCA tumor burden at day 32 compared with the control AIN93G cohort. E, Kaplan–Meier survival curve of the RENCA 21% and 7% diet cohorts ± anti-PD-1. Results are presented as the mean ± SE. Statistical significance was determined using Student t test with Welch correction. *, \( P < 0.05 \); **, \( P < 0.01 \).
alterations (47). We observed a significant decrease in the relative abundance of multiple Ruminococcaceae and Prevotellaceae family OTU in the 80MR cohort. Additionally, we observed a significant increase in the presence of members of the Lactobacillaceae family (Supplementary Fig. S9D–S9F).

Taken together, these results provide new insights on the impact of AA restriction and underlying pathways involved in macrophage polarization and function (Supplementary Fig. S10).

Discussion

In this study, we report that a reduction in dietary protein or methionine/cystine intake significantly reduces the polarization of M2-type macrophages. Importantly, these dietary interventions also affect M2-type macrophage functions and TME infiltration in vivo. M/CR was sufficient to recapitulate the antitumor effect observed in our previous study with dietary protein restriction (2, 15). Based on our cystine/methionine restricted diet studies, we observed an inhibition of tumor growth which was significantly enhanced with immunotherapy in the protein restriction setting. We also found a resulting shift away from an immunosuppressive TME, one characterized by transition of an M2- to M1-type macrophage response, accompanied by modulation of the mTOR/transsulfuration/xCT/cytokine production pathway. Models with high infiltration of M2-type macrophages were chosen to reflect clinical characteristics of advanced-stage kidney and prostate cancer. Recent studies suggest the role of macrophages in promoting prostate-to-bone metastases and underscore a strong correlation between TAM density and preclinical outcome in advanced-stage prostate cancer patients (48, 49).

To examine the effect of AA alterations on key functional macrophage pathways, we hypothesized that, because macrophages are driven by circulating stimuli, including available nutrients, they may also be affected by decreased levels of total protein. AA composition of the diet appears to play an
essential role in the effects described here, as the presence of varying AAs are known to feed into central nutrient-sensing pathways, including the mTOR and transsulfuration pathways (17, 44, 45). Moreover, these pathways are critical to the response of the innate immune system to diseases, including infections and cancer (12, 17). Our study suggests that AA restriction, mimicking reduced dietary protein levels in the host, leads to significant alterations in nutrient-sensing and macrophage-polarizing pathways. We observed inhibition of the M2-driving, nutrient-sensing mTOR, Nrf2, and PI3K/AKT pathways, which have been linked to increased production of protumor or decreased production of proinflammatory cytokines (38, 46, 50). Further, we observed an increase in Nfat5 expression, which is intricately linked to increased production of proinflammatory, M1-type cytokines (38), and an increase within the Atf4/Xct axis followed by a moderate increase in ROS.

An evolving concept that contributes to the understanding of macrophage polarization is that the ROS spectrum affects macrophage status, where those macrophages with low ROS are of an M2 status, moderate ROS are of M1 status, and high ROS leads to apoptosis (33). Given the importance of macrophage polarization and function in cancer progression (8), we propose that dietary protein/AA restriction contributes to a pivotal shift in the function of tumor-infiltrating macrophages or TAMs. Our data define, for the first time to our knowledge, a comprehensive outline of the association of multiple nutrient-sensing pathways, their response to AA restriction, and the subsequent impact on macrophage polarization. Macrophages have been implicated in the progression and aggressiveness of prostate and renal carcinomas (9). Through the use of a highly immunogenic, syngeneic mouse model of prostate cancer, we determined that dietary M/CR led to significant proinflammatory changes in the tumor-infiltrating immune cells. Thus, we propose that these dietary modifications, in particular protein restriction, may enhance the response of the host system to immunotherapies.

Accumulation of myeloid cells within the TME, tumor escape, and the magnitude of T-cell responsiveness have recently been connected to the polyamine biosynthesis pathway (40). Here, we show preliminary evidence linking polyamine biosynthesis to macrophage polarization in the TME. Importantly, direct blockade of polyamine biosynthesis via inhibition of ODC1 with DFMO resulted in the same increase in M1-type macrophage polarization and reduction of M2-type macrophages that we saw with dietary restriction of methionine. Our data indicate a potential causal link between dietary MR and the polyamine synthesis pathway, which is known to promote proinflammatory M1-type macrophage responses in the TME (40). Taken together, these results open potential future directions for combining dietary modifications with polyamine blockade in the presence of IT regimens.

Figure 6.
AA restriction modulates the molecular programming of macrophage polarization: A, relative expression of cystine and glutamine transporter xCT in M1 and M2 phenotypes in response to AA restriction. B, Normalized Nrf2 gene expression. C, Protein-level expression of mTOR pathway downstream target, pS6. Top, Densitometry analysis of the phosphorylated S6 seen in the Western blot. Bottom, Western blot analysis of mTOR activation marker pS6. D, Protein-level analysis of negative regulator of Atf4, eIF2α. (top) densitometry analysis of the Western blot (bottom). E, Quantitative analysis from Enzo ROS-ID Total ROS Detection Kit in M1 and M2 conditions (n = 9). F, Normalized gene-expression analysis of proinflammatory cytokine Ccl2. All qRT-PCR results are normalized to GAPDH and run in triplicate for n = 3 independent experiments. Results are presented as the mean ± SE. Statistical significance was determined using Student t test with Welch correction. *P < 0.05, **P < 0.01; ***P < 0.001; ****P < 0.0001. Legend: a, control media. b, 1/2M, c, 1/2M 1/3C; 1. control M1, 2. control M2, 3. 1/2M M1, 4. 1/2M M2, 5. 1/2M 1/3C M1, 6. 1/2M 1/3C M2, 1. 1/2M M1 2. 1/2M 1/3C M1 3. 1/2M M2 4. 1/2M 1/3C M2.
Nevertheless, we believe that our observations have a significant carryover effect that temporary diet modifications may prime the immune system during the initial treatment with immunotherapy. Therapeutic targeting of macrophages achieved by a dietary modification, rather than a pharmacologic approach, represents an exciting opportunity for the clinical testing of this supportive tool to affect "inflamed" tumors and enhance the efficacy of current immunotherapies.

Disclosure of Potential Conflicts of Interest

M. J. Ciesielski is an employee of and holds ownership interest (including patents) in MimiVax, LLC. No potential conflicts of interest were disclosed by the other authors.

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