The Acute Effects of 5 Fluorouracil on Skeletal Muscle Resident and Infiltrating Immune Cells in Mice

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Abstract

Background: 5 fluorouracil (5FU) has been a first-choice chemotherapy drug for several cancer types (e.g. colon, breast, head & neck); however, its efficacy is diminished by patient acquired resistance and pervasive side effects. Leukopenia is a hallmark of 5FU; however, the impact of 5FU-induced leukopenia on healthy tissue is only becoming unearthed. Recently, skeletal muscle has been shown to be impacted by 5FU in clinical and preclinical setting and weakness and fatigue remain among the most consistent complaints in cancer patients undergoing chemotherapy. Monocytes, or more specifically macrophages, are the predominate immune cell in skeletal muscle which regulate turnover and homeostasis through both the removal of damaged or old materials and coordinate repair and remodeling. Whether 5FU-induced leukopenia extends beyond circulation toimpact resident and infiltrating skeletal muscle immune cells had not been examined. The purpose of the study was to examine the acute effects of 5FU on resident and infiltrating skeletal muscle monocytes and inflammatory mediators.

Methods: Male C57BL/6 mice were given a physiologically translatable dose (35mg/kg) of 5FU, or PBS, i.p. once daily for 5 days to recapitulate 1 dosing cycle.

Results: Our results demonstrate that 5FU reduced circulating leukocytes, erythrocytes, and thrombocytes while inducing significant body weight loss (>5%). Flow cytometry analysis of the skeletal muscle indicated a reduction in total CD45+ immune cells with a corresponding decrease in total CD45+CD11b+ monocytes. There was a strong relationship between circulating leukocytes and skeletal muscle CD45+ immune cells. Skeletal muscle Ly6c^{High} activated monocytes and M1-like macrophageswere reduced with 5FU treatment while total M2-like CD206+CD11c-macrophageswere not changed with 5FU. Interestingly, 5FU reduced bone marrow CD45+ immune cells and CD45+CD11b+ monocytes.

Conclusions: Our results demonstrate that 5FU induced body weight loss and decreased skeletal muscle CD45+ immune cells in associated with a reduction in infiltrating Ly6c^{High} monocytes. Interestingly, the loss of skeletal muscle immune cells occurred with bone marrow cell cycle arrest.Together our results highlight that skeletal muscle is sensitive to the cytotoxic effects of 5FU which disrupts both circulating and skeletal muscle immune cells.

Background

The increase in 5-year survival rate among cancer patients has increased focus on quality of life to improve patient outcomes(1). In addition to cancer-associated wasting and functional decrements, the most commonly prescribed chemotherapies have pervasive off-target effects.(2-5) 5 fluorouracil (5FU) has been the first-choice chemotherapy drug for several cancer types for many years(6-9);however, 5FU negatively impactsthe gastro-intestinal system(2, 4, 10), cardiovascular systems(3, 11), hematopoietic system(12-14), and has recently been shown to directly disrupt skeletal muscle mass and function(15-17).Disruptions to skeletal muscle homeostasis contributes to functional dependency and poor treatment outcomes and ultimately leads to increased healthcare costs and decreased survival(15). Currently, there are no FDA approved therapies for chemotherapy-induced cachexia despite the importance ofskeletal muscle mass and function maintenance in sustaining 5FU's therapeutic efficacy and patient quality of life(16, 18). This is not entirely surprising given that very little is known about the mechanisms responsible for 5FU-induced skeletal muscle dysfunction. Thus, identifying the factors driving chemotherapy-induced skeletal muscle dysfunction is critical to developing effective interventional therapies.

Despite 5FU-induced leukopenia remaining a hallmark of treatment(10, 19), investigations intothe impact of 5FU on skeletal muscle have been largely focused on metabolism(15, 20),Notably, there is a dearth of evidence on the influence of 5FU on skeletal muscle inflammation – a process that is known to play a role in skeletal muscle homeostasis(21).Indeed, inflammation can play a paradoxical role in skeletal muscle homeostasis. During normal conditions pro-inflammatory cytokines are required to balance anabolism and catabolism and to maintain normal myogenesis process. However, during disease conditions, pro-inflammatory cytokines caninduce catabolic pathways that impair skeletal muscle integrity and function(22). To date,our understanding of 5FU-induced inflammatory changes is limited to circulating inflammatory cytokines and intrinsic inflammatory signaling. Additionally, the available studies highlight equivocal results showing increased circulating interleukin (IL) 6, tumor necrosis factor α (TNFα), monocyte chemoattractant protein (MCP) 1(23-25),with reduced or unchanged skeletal muscle inflammatory protein expression(15, 20).Given theimportance of skeletal muscle mass and function maintenance to quality of life in chemotherapy patients along with the well-documented effects of inflammation on skeletal muscle homeostasis,it is important to assess inflammatory mediators asa potential target for chemotherapy-induced skeletal muscle dysfunction(12-14, 26-28).

The lack of evidence on 5FU associated perturbations in skeletal muscle inflammation is consistent with a scarcity of literature on 5FU effects on skeletal muscle immune cells. Monocytes, or more specifically macrophages, are the most abundant skeletal muscle immune cell which function to regulate tissue turnover and homeostasis(29).Targeting macrophages is emerging as a potential key regulator of chemotherapeutic efficacy given the importance of tumor associated macrophages (TAM) in tumorigenesis, tumor vascularization, and local immunosuppression(30); however, the effects of 5FU on skeletal muscle macrophages is largely unexplored. Resident skeletal muscle monocytes are classically characterized as CD11b+Ly6c^{Low} monocytes and F4/80+CD11c-CD206- (quiescent – M0)macrophages(29, 31).CirculatingCD11b+Ly6C^{High}activated monocytes,recruited by MCP-1,extravasate the muscle(32, 33) and either remain CD11b+Ly6C^{High}or differentiate to F4/80+CD11c+CD206- pro-inflammatory, pro-phagocytic (M1-like) macrophages which secrete pro-inflammatory cytokines, IL-6, IL-1β, TNFα, and interferon (IFN) γ(34, 35).These M1-like macrophages will thendown regulate CD11c expression and increase CD206+to reflect a more anti-inflammatory, pro-fibrotic(M2-like)macrophage which secrete anti-inflammatory cytokine IL-10 and pro-fibrotic cytokine transforming growth factor (TGF) β(36-38).Proper balance of these immune cell phenotypes and maintenance of immune cell number are vital for skeletal muscle homeostasis.Thus, determination of 5FU's effects on skeletal muscle immune populations is essential for the development of effective treatment strategies.

Skeletal muscle immune cell depletion has been demonstrated to delay recovery and disrupt extracellular matrix remodeling leading to fibrosis, weakness, and metabolic homeostatic imbalance(39, 40).While results pertaining to intrinsic skeletal muscle inflammatory signaling with several chemotherapies are equivocal, leukopenia has been well established(12-14, 26-28).The overall purpose of the current studywas to investigate the acute effects of 5FU on resident

and infiltrating skeletal muscle monocytes and inflammatory mediators.We hypothesized that an acute dosing regimen of 5FU wouldde plete circulating and skeletal muscle monocytesand reduce associated inflammatory cytokines consistent with systemic leukopenia. Our results demonstrate that 1 cycle of 5FU was sufficient to induce significant body weight loss and leukopenia associated with a loss of total skeletal muscle immune cells and a reduction in select inflammatory mediators. Additionally, we show 5FU induced bone marrow cell cycle arrest which is likely to contribute to the observed loss of infiltrating skeletal muscle monocytes.

Methods

Animals

Eighteen male C57BL/6 mice were purchased from Jackson Laboratories at 4 wks of age and housedin the Department of Laboratory Animal Resources at the University of South Carolina. Mice were either group housed (n=12) or singly housedto measure food intake (n=6) and kept on a 12:12-h light-dark cycle. Animals were placed on a purified AIN-76A(BioServ, Frenchtown, NJ, USA; catalog#:F1515) diet for 5wks prior to any experimental procedures. Body weights were measured weekly, and animals were monitored for signs of distress. Animals were given food and water ad libitum throughout the duration of the study. All animals were fasted 5 hrs prior to tissue collection. Mice were anesthetized with isoflurane and hindlimb muscles, select organs, and both femurs were carefully dissected, weighed, and either snap frozen in liquid nitrogen or placed in the appropriate buffers for flow cytometry analysis. All animal experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee.

Experimental Design

Male mice (n=18) were purchased from Jackson laboratories at 4 wks of age, acclimated to the new facilities for 5wks, and given a purified AIN-76A diet for 5 wks prior to the start of the study. At 14 wks of age mice were randomized into 2 groups, Control (n=9) and 5FU (n=9). 5FU was solubilized in PBS at 3.5 mg/mL and administered to the mice at 35 mg/kg i.p. once daily for 5 days. This dosing regimen has been previously shown to be comparable to clinical doses and recapitulates 1 cycle of chemotherapy(10, 11).Control mice received a PBS injection. Tissue was collected and the animals were euthanized 24hrs following the final injection.

Blood analysis

Blood was collected at euthanasia via the inferior vena cava,placed in an EDTA coated vacutainer (VWR, Suwanee, GA, USA; catalog#:454428) and stored briefly on ice until analysis. A complete blood count was performed using the VetScan HMT (Abaxis, Union City, CA) for determination of white blood cells (WBCs), lymphocytes (LYM), monocytes (MON), neutrophils (NEU), red blood cells (RBCs), Hemoglobin (HGB), Hematocrit (HCT), and platelets (PLT).

Flow cytometry

Both quadriceps were excised, minced in Dulbecco's Modified Eagle Medium(DMEM), and cells were extracted using the skeletal muscle dissociation kit (MiltenyiBiotec, Auburn, CA; cat#; 130-098-305) following the manufacture's instruction. Both quadriceps were pooled to obtaina sufficient number of cells for each analysis without pooling animals (n=9/group). Skeletal muscle cells were suspended in flow buffer (0.5% BSA, 2mM EDTA, PBS). Following hindlimb muscle excision, both femurs (n=5/group) were cleaned and placed in ice cold PBS. The epiphysis of the femurs wasremoved, and the bone marrow was flushed with PBS using a 26G syringe. Cells were then passed through a 70-µm filter and suspended in flow buffer (2%FBS-PBS). Red blood cell lysis was performed with 20 second hypotonic solution (0.2% NaCl) treatment followed by hypertonic (1.6% NaCl) cessation.This method has been shown to reduce disturbances to cell surface markers compared to alternative RBC lysis buffers.(41)Both skeletal muscle and bone marrow cells were blocked with Fc-block against CD16 and CD32 in their respective flow buffers. Cells were then incubated with fluorescently labelled antibodies against CD45 (PE/CY7), CD11b (APC), Ly6c (PerCP/Cy5.5), F4/80 (FITC), CD11c (APC/Cy7), and CD206 (PE). Cells were measured using a FACS Aria II and analyzed using FlowJo V10.6.2 (BD Biosciences, Ashland, Oregon).Prior to cellular analysis, all colors were compensated using Invitrogen UltraCompeBeads^{TM} Compensation Beads (Life technologies, Carlsbad CA). A total of $5x10^5$skeletal muscle cells and $3x10^5$ bone marrow cells were analyzed.

RNA isolation and RT-PCR

RNA isolation, cDNA synthesis, and real-time PCR were performed as previously described(10) using reagents from Applied Biosystems (Foster City, CA, USA). Briefly, RNA was extracted from the gastrocnemius using the TRIzol/isopropanol/chloroform procedure (Life Technologies, GIBCO-BRL, Carlsbad, CA). RNA sample quality and quantities were verified using aNanodrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) and determined to be of good quality based on A260/A280 values (> 1.8) prior to cDNA synthesis using High capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA). Probes for MCP-1, IL-6, IL-1β, IL-10, TNF-α, IFNγ, CD11c, CD206, F4/80, and CD68 as well as housekeeping genes Hmbs, B2M, TBP, H2afv, and 18s were purchased from Applied Biosystems (Foster City, CA, USA). Quantitative RT-PCR analysis was carried out as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA) using Taq-Man Gene Expression Assays on a Qiagen Rotor-Gene Q. Data were normalized to vehicle treated controls and compared to fivereference targets (Hmbs, B2M, TBP, H2afv, and 18s), which were evaluated for expression stability using GeNorm.(42)

Statistics

Values are presented as means ± standard error of the mean (SEM). Student t-tests were performed to determine the differences between 5FU and Control for all endpoint measurements. A repeated measures two-way ANOVA was used to determine a difference in body weight change and food intake (treatment x time). Post hoc analysis were performed with student Newman-Keuls methods. A Bartlett's test was used to determine significantly different standard deviations. Significance was set at $p \leq 0.05$.

Results

Animal characteristics

Body weights were monitored daily during the treatment period and shown as a relative change (%) from Day 0. 5FU treated mice exhibited body weight loss between day 0 and Day 5 (effect of time; p<0.0001) and had reduced % body weight between days 2-5 (2-8%) compared to controls(p<0.0001) (Figure 1A). 5FU reduced the overall average daily food intake (g/day) by 20.5% (p=0.006) compared to controls(Figure 1B). Despite the reductions in body weight and food intake, there were no observed differences between 5FU and controlsin several hindlimb muscle weights (Figure 1C). Spleen weight was decreased22.6% (p<0.0001) with 5FU (Figure 1D) which is further supported by a 46.4% (p=0.001) decrease in circulating leukocytes (Figure 1E). More specifically,circulating lymphocytes and neutrophilswere reduced with 5FU by 33.3% (p=0.006) and 83.5% (p=0.002), respectively, with no apparent change in circulating monocytes (Figure 1E).5FU reduced circulating red blood cells (RBC) by 16.2% (p=0.002) with 20.8% (p=0.0003) and 18.8% (p=0.0002) reductionsin hemoglobin (HGB) and hematocrit (HCT), respectively (Figure 1F). Additionally, 5FU decreasedplatelets (PLT)by 62.6% (p<0.0001; Figure 1F).

The effect of 5FU on Skeletal Muscle Monocytes

Cells isolated from the quadriceps underwent the following gating procedures, which was previously described.(38) Cells were first gated for “non-debris” by plotting SSC-A x FSC-A (Figure 2A). Cells were then gated for single cells by plotting SSC-W x SSC-H (Figure 2B) and then FSC-W x FSC-H (Figure 2C). Immune cells were then gated from “non-debris”, “SSC singlets”, and “FSC singlets” by plotting SSC-A by CD45. CD45+ cells were considered all immune cells and were quantified as a % of singlets (Figure 2D) and total number of immune cells (Table 1). 5FU treatment resulted in a 35.5% decrease (p=0.003) in the relative quantity of CD45+ immune cells (Figure 2E), and a 46.9% decrease in total CD45+ immune cells (Table 1). CD45+ immune cells were further gated with CD11b and CD45+CD11b+ cells were classified as monocytes and were quantified as a % of CD45+ cells (Figure 2F) and total number of monocytes (Table 1). The relative abundance of monocytes within CD45+ cells was not changed with 5FU treatment (Figure 2G); however, total monocytes were reduced by 47.0% with 5FU.CD45+CD11b+ cells were further gated with F4/80 and CD45+CD11b+F4/80+ cells were classified as macrophages and were quantified as a % of CD45+CD11b cells (Figure 2H) and total number of macrophages (Table 1). 5FU decreased the relative abundance of macrophages by 19.2% within CD45+CD11b+ monocytes; however, this did not reach statistical significance (p=0.07; Figure 2I).5FU reduced total macrophage count by 56.8% (Table 1). Last, there was a strong correlation between circulating leukocytes and skeletal muscle CD45+ immune cells (R=0.75; p=0.003), CD11b+ monocytes (R=0.69; p=0.002), F4/80+ macrophages (R=0.67; p=0.002), and Ly6c^{High} infiltrating monocytes (R=0.67; p=0.008) in all mice.

Skeletal muscle inflammatory geneexpression

RNA was extracted from the gastrocnemius which shares a similar myofibrillar myosin heavy chain isoform expression as the quadriceps and is similarly a prime mover. There was no difference in expression of total macrophage markers CD68 or Emr1 (F4/80) with 5FU treatment (Figure 3A). Additionally, there was no difference in M1-like macrophage gene Itgax (CD11c) or M2-like macrophage gene Mrc1 (CD206) with 5FU (Figure 3A). 5FU reduced pro-inflammatory cytokines, IL-1β and IFNγ, 95% (p=0.009) and 75% (p=0.01), respectively, while IL-6, TNFα, and MCP-1 were not changed (Figure 3B). There were no differences in anti-inflammatory cytokines IL-10 and TGFβ with 5FU (Figure 3C).

The effect of 5FU on Resident and Infiltrating Skeletal Muscle Monocytes and Macrophages

Given the decrease in pro-inflammatory cytokines IL-1β and IFNγ, we sought to understand the phenotype of skeletal muscle monocytes. Similar to Figure 2, cell singlets were gated for CD45+CD11b+ followed by Ly6C to understand the effects of 5FU on infiltrating monocytes. Ly6c^{High} cells were classified as infiltrating monocytes and were quantified as a % of CD45+CD11b+ cells (Figure 4A) and total number of activated monocytes (Table 2). 5FU decreased the relative abundance of Ly6c^{High}infiltrating monocytes by 49.9% (p=0.02) within CD45+CD11b+ monocytes (Figure 6B). Also, total Ly6c^{High}infiltrating monocytes were reduced by 73.0% with 5FU, but this did not achieve statistical significance (p=0.06; Table 2). Total Ly6c^{Low}, resident monocytes, were reduced by 38.6% with 5FU (Table 2).Given that the total number of macrophages were reduced with 5FU(Table 1), we examined if there were changes in macrophage phenotype by measuring CD11c (M1-like) and CD206 (M2-like) from parent CD45+CD11b+F4/80+ macrophages (Figure 4C). There were no observed changes in the relative abundance ofM1-like, M2-like, M1-M2 transitional, or M0 macrophages with 5FU treatment (Figure 4D-G); however, 5FU decreased total number of M1-like (CD11c+CD206-) by 70.7%, M1-M2 transition (CD11c+CD206+) by 63.6%, and M0 (CD11c-CD206-) by 57.0% (Table 2). The total number of M2-like macrophages (CD11c-CD206+) cells were not changed by 5FU (Table 2).

The effects of 5FU on the Bone Marrow

In order to further understand the effects of 5FU on circulating and infiltrating monocytes, we examined 5FU’s impact on bone marrow cells (Figure 5). Bone marrow isolates were obtained from both femurs of 5 mice/group. Cell gating of bone marrow cells was performed as described for data in Figure 2 (Figure 5A-D). 5FU decreased the relative abundance of CD45+ immune cells by 12.9% (p=0.03; Figure 5E) and total CD45+ immune cells by 19.3% (Table 3), but the reduction in total CD45+ immune cells did not reach statistical significance (p=0.096). CD45+ cells were further gated with CD11b and CD45+CD11b+ cells were considered monocytes (Figure 5F) and were quantified as a % of CD45+cells (Figure 5G) and total number of monocytes (Table 3).5FU treatment reduced the relative abundance of bone marrow monocytes by 50.3% (p=0.0002) within total CD45+ immune cells (Figure 5G) and reduced total monocytes by 60.7%. CD45+CD11b+ cells were further gated with Ly6C and CD45+CD11b+Ly6C^{High} cells were considered activated monocytes (Figure 5H) and were quantified as a % of CD45+CD11b+ cells (Figure 5G) and total number of activated monocytes (Table 3). 5FU treatment had no apparent effect on the relative abundance of bone marrow activated monocytes within total CD45+CD11b+monocytes (Figure 5I); however, the total number of activated monocytes was reduced by 53.2% (Table 3). Additionally, 5FU induced cell cycle arrest in the bone marrow (Figure 6). 5FU increased the relative abundance of cells in the G1/G0 cell cycle phase by 12.0% (p=0.009) and decreased S and G2/M cell cycle phases by 82.4%(p<0.0001) and 69.1% (p<0.0001), respectively (Figure 6B).

Discussion

5FU has been the first-choice chemotherapy drug for several cancer types;however, its efficacy is diminished by patient acquired resistance and pervasive side effects contributing to reduced life quality and poor treatment outcomes(6-9).Given 5FU's deleterious effects on circulating leukocytes, the purpose of ourstudywas to investigate the acute effects of 5FU on resident and infiltrating skeletal muscle monocytes and inflammatory mediators in addition to examining the effects of 5FU on circulating and bone marrow immune cells. Our results extend previous studies to identify that 1 cycle of a clinically translatable dose of 5FU significantly reduced CD45+ immune cells and infiltrating/activated CD11b+Ly6C^{High} monocytes in skeletal muscle that was associated with a decrease in select skeletal muscle inflammatory mediators. Additionally, the reduction in skeletal muscle and circulating immune cells wasaccompanied by a reduction in bone marrow monocytes and an increase in cell cycle arrest. These resultsidentify novel off-target effects of 5FU on skeletal muscle and the skeletal muscle microenvironment.

Our understanding of chemotherapy-induced body weight and function loss, termed cachexia, has improved over the last decade(15, 43, 44).Our investigation of the acute (1 cycle) effects of 5FU demonstrated that 5FU induced clinically relevant body weight loss (>5%)(45), which was accompanied with signs of anorexia, but not skeletal muscle mass loss. Others have demonstrated that 5 weeks of 5FU combination therapy, Folfiri (leucovorin, 5FU, Irinotecan), demonstrated decreased body weight and lean mass over time, with corresponding reductions in several hindlimb weights(15).Interestingly, mice given 5 weeks of Folfox (leucovorin, 5FU, oxaliplatin) rather than Folfiri maintained body weight and lean mass, and only showed reduced quadriceps weight. Additionally, similar to the doxorubicin effects on skeletal muscle(43, 46),only Folfiri reduced skeletal muscle specific force (strength per muscle unit area) – which may occur through several mechanisms including fibrosis(15).Together this evidence suggests that significant muscle mass loss only occurs after sustained 5FU treatment(15, 47),given that 1 week of 5FU was unable to reduce hindlimb muscle weight. We then hypothesize that a loss of water weight is likely to contribute to the observed body weight loss as anorexia and dehydration with 5FU has been reported(48, 49).However, pathologies that occur early in the treatment regimen may provide insight into the etiology of muscle mass and strength losses with 5FU. To this end, 5FU-induced anemia and leukopeniaare likely to contribute to the observed functional pathologies that occur late with 5FU treatment.

The role of immune cells, particularlymacrophages, in skeletal muscle regeneration, repair, and remodeling has been well characterized(29); however, chemotherapy's effects on these processes is not well known. Following skeletal muscle insult (e.g. damage, ischemia, exercise), there is an initial influx of neutrophils which in turn recruit naïve monocytes primarily through the release of MCP-1(29, 33). We have previously shown that 5FU induced circulating MCP-1 after 14 days of treatment, which was associated with reduced voluntary physical activity(23). The monocytes recruited by MCP-1 are primarily recruited as CD11b+Ly6C^{High} monocytes which can either remain as such or differentiate and polarize to a pro-inflammatoryM1-like F4/80+CD11c+CD206- macrophage(34, 50, 51).Following an acute 5FU regime (1 week) we document a reduction in total and relative Ly6C^{High} monocytes as well as totalM1-like F4/80+CD11c+CD206-macrophages in skeletal muscle despite no changes in skeletal muscle pro-inflammatory MCP-1, IL-6 and TNFα levels. However, we did observe decreased expression of pro-inflammatory genes associated with M1-like macrophages, IL-1β and IFNγ, but on the other hand did not observe corresponding changes to total M1-like macrophage cell surface marker, Itgax, more commonly known as CD11c(52).These discrepancies between the flow cytometry and gene transcription require additional work and thus, interpretations should be taken with caution; however, flow cytometry remains the gold standard for the assessment of immune cells, and it appears evident that 5FU has deleterious effects on the pro-inflammatory monocytes and macrophages.A loss of pro-inflammatory or phagocytic M1-like macrophages could negatively impact skeletal muscle remodeling and repair(50). Chemotherapeutic doxorubicin has been shown to blunt the pro-inflammatory response following exercise which mitigated the muscle's response to exercise(53). Furthermore, while repeated muscular contractions were able to improve muscle mass in cancer patients undergoing treatment, patients did not obtain the functional and metabolic improvements that has been previously seen with exercise(54, 55).While chemotherapeutics 5FU and doxorubicin mechanisms of action differ, we can still glean potential mechanisms and clinical manifestations. To the best of our knowledge, we are the first to identify that 5FU disrupts skeletal muscle's pro-inflammatory immune cell environment. It is also important to note that these cell surface markers and the M1/M2 dichotomous classification of macrophages does not properly reflect the true diversity and nature of resident/infiltrating macrophages and should again be interpreted cautiously(29, 34, 56, 57).

Tissue resident macrophages are classically CD206+ anti-inflammatory, pro-fibrotic surveying macrophages;(34, 37, 58, 59) however, macrophages are plastic and as skeletal muscle repair progresses theinfiltratedM1-like F4/80+CD11c+ macrophages can reduce the gene expression and release of pro-inflammatory mediators and become more phenotypically M2-like to promote extracellular matrix remodeling and angiogenesis.(60-62)Others have proposed that resident macrophages are predominantly M0 (CD11c-CD206-) which are self-maintained, proliferate, and polarize to an M1-like phenotype upon activation during the initial stages of injury repair(29, 59). Regardless, our results demonstrate that the relative phenotype of skeletal muscle macrophages is not changed by 5FU treatment; however, the total number of M1-like (CD11c+CD206-), M0-like (CD11c-CD206-), and M1-M2-like transitional macrophages were reduced with 5FU while M2-like macrophages appear spared from 5FU's cytotoxicity – at least following 1 week of 5FU. Additionally, anti-inflammatory IL-10, pro-fibrotic TGFβ, and M2-like macrophage cell surface marker Mrc1, commonly known as CD206, gene transcription were not changed by 5FU treatment. The potential for 5FU to target M1-like macrophages rather than M2-like, points to a pro-fibrotic skeletal muscle microenvironment. 5FU combination therapy Folfiriwas shown to reduce skeletal muscle specific force (force per unit area); however, neither fibrosis nor an increase in fibrotic genes (TGF-β associated ligands) were apparent(15). Therefore, it is likely thatthese pro-fibrotic M2-like cells remain at a physiological abundance during 5FU treatment and may not be contributing to a skeletal muscle pathology directly.Interestingly,TAMs phenotypically reflect M2-like macrophages promoting immunosuppression, fibrosis, and angiogenesis, within the tumor microenvironment and have been associated with 5FU acquired resistance(63). The potential for M2-like macrophages to be protected against 5FU requires significant attention in the cancer domain.

Chemotherapy has been shown to mitigate the inflammatory response with exercise(53, 64), induce leukopenia/cytopenia(19), and disrupt cardiacmacrophage infiltration(65). To the best of our knowledge, this is the first study to demonstrate that chemotherapeutic 5FU has deleterious effects on immune cell abundance in otherwise healthy uninjured skeletal muscle. The absolute reduction in macrophage number rather than relative changes in

abundance remains relevant given the physiological importance of the overall immune response in repair and remodeling(59, 66-70). The mean age of cancer patients is ~65yrs and overlapping sarcopenic and cachectic factors along with chemotherapy may contribute to disrupted skeletal muscle immune regulation(71).Disrupted skeletal muscle repair associated with changes in macrophages has been reported with aging(38), cancer(70), and chemotherapy(53).The effects of aging on skeletal musclemacrophages has demonstrated that reloading aged skeletal muscle had a blunted hypertrophy response associated with a lower number of M1-like macrophages at baseline and blunted M1-like macrophage infiltration (early) and M2-likemacrophage transition (late)(38).Surprisingly, while inflammation is a hallmark of cancer cachexia associated with muscle weakness and fatigue(72, 73), total macrophage number was reduced in damaged muscle of C26tumor-bearing mice compared to a non-cachectic tumor-bearing control(70). Additionally, macrophages were shown to regulate skeletal muscle signal transducer and activator of transcription 3 (STAT3) – downstream target of IL-6 and key regulator of skeletal muscle mitochondrial homeostasis and proteostasis(73-77)– during pancreatic cancer cachexia(78).Further work is needed to understand these potentially overlapping mechanisms with cancer and chemotherapy on skeletal muscle immune cells.

Chemotherapy's effects on systemic inflammatory mediators(2, 4, 10, 79) and intrinsic skeletal muscle inflammatory signaling(12-14, 26-28) are continuing to be unearthed; however,our study is the first to identify that 5FU-induced leukopenia extends beyond circulation to impact the skeletal muscle microenvironment.Our results indicate that 5FU's toxic effects on skeletal muscle leukocytes are not necessarily specific to monocytes shown by no change in both circulating monocyte count or relative abundance of skeletal muscle CD11b+ monocytes within the CD45+ population. This is not to say that monocytes aresparedfrom 5FU as the total number of skeletal muscle monocytes are reduced. Bone marrow CD11b+ monocytes and the relative abundance of infiltrating $Ly6C^{High}$ monocytes in skeletal muscle were reduced with 5FUwhich is supported by the established deleterious effects of 5FU on circulating leukocytes and the hematopoietic system(8, 10, 27). In conjunction with previous studies, our results support that 5FU's toxicity is predominantly associated with pro-inflammatory mediators extending beyond the hematopoietic system to impact the skeletal muscle microenvironment. Another potential mechanism for the observed impact of 5FU on skeletal muscle immune cells is the potential forreduced proliferation of pro-inflammatory macrophages within the muscle microenvironment. Given that circulating and skeletal muscle monocytes/macrophages are not proportionally reduced it is possible that 5FU increased maturation of monocytes within the skeletal muscle as well as increased proliferation of M2-like macrophages.

Conclusions

Understanding chemotherapy's off-target effects will allow for improvements to treatment efficacy aimed at increasing cancer patient survival and quality of life. Our novel finding that chemotherapeutic 5FU depletes skeletal muscle immune cells and infiltrating monocytes provides insight into the skeletal muscle microenvironment that can contribute to weakness, fatigue, and treatment intolerance(16). We provide evidence to suggest that 5FU reduced circulating and skeletal muscle leukocytes through disrupting thehematopoietic system by inducing cell cycle arrest in the bone marrow. Future studies are needed to understand the long-term implications of this loss of immune cells and if chronic 5FU exposure exacerbates this immune dysregulation. Furthermore, additional work is needed to determine if mitigating the loss of immune cells can improve skeletal muscle function following repeated cycles of 5FU.

List Of Abbreviations

5FU – 5 fluorouracil

BSA – bovine serum albumin

CD – cluster of differentiation

DMEM - Dulbecco's Modified Eagle Medium

EDTA – Ethylenediaminetetraacetic acid

FBS – fetal bovine serum

FSC – forward scatter

HCT – Hematocrit

HGB – Hemoglobin

IFN – interferon

IL – Interleukin

Ly6c – lymphocyte antigen 6c

LYM – lymphocyte

MCP – monocyte chemoattractant protein

MON – monocyte

NEU – neutrophil

PBS – phosphate buffered saline

PLT – platelets

RBC – red blood cell

SEM – standard error of the mean

SSC – side scatter

STAT – signal transducer and activator of transcription

TAM – tumor associated macrophage

TGF – transforming growth factor

TNF – tumor necrosis factor

WBC – white blood cell

Declarations

Ethics Approval

All animal experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee.

Consent for publication

Not applicable

Availability of data and material

All data generated and collected are included in the published work. All materials and reagents are commercially available.

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Competing Interests

The authors declare no conflicts of interest.

Author Contributions

BNV, JAC,KJV, DF, and EAM conceived and designed the experiments. BNV and ATS performed experiments. BNV prepared the figures. BNV drafted the manuscript. BNV, ATS, KJV, JAC, DF, and EAM edited, revised, and approved final version of the manuscript.

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Not applicable

References

1. Curt GA, Breitbart W, Cella D, Groopman JE, Horning SJ, Itri LM, et al. Impact of cancer-related fatigue on the lives of patients: new findings from the Fatigue Coalition. Oncologist. 2000;5(5):353-60.
2. Lee CS, Ryan EJ, Doherty GA. Gastro-intestinal toxicity of chemotherapeutics in colorectal cancer: the role of inflammation. World J Gastroenterol. 2014;20(14):3751-61.
3. Polk A, Vistisen K, Vaage-Nilsen M, Nielsen DL. A systematic review of the pathophysiology of 5-fluorouracil-induced cardiotoxicity. BMC Pharmacol Toxicol. 2014;15:47.
4. Ribeiro RA, Wanderley CW, Wong DV, Mota JM, Leite CA, Souza MH, et al. Irinotecan- and 5-fluorouracil-induced intestinal mucositis: insights into pathogenesis and therapeutic perspectives. Cancer Chemother Pharmacol. 2016;78(5):881-93.
5. Iacovelli R, Pietrantonio F, Palazzo A, Maggi C, Ricchini F, de Braud F, et al. Incidence and relative risk of grade 3 and 4 diarrhoea in patients treated with capecitabine or 5-fluorouracil: a meta-analysis of published trials. Br J Clin Pharmacol. 2014;78(6):1228-37.
6. McQuade RM, Stojanovska V, Bornstein JC, Nurgali K. Colorectal Cancer Chemotherapy: The Evolution of Treatment and New Approaches. Curr Med Chem. 2017;24(15):1537-57.
7. Giuliani J, Bonetti A. The Pharmacological Costs of Complete Liver Resections in Unselected Advanced Colorectal Cancer Patients: Focus on Targeted Agents. A Review of Randomized Clinical Trials. J Gastrointest Cancer. 2016;47(4):341-50.

8. Lee JJ, Beumer JH, Chu E. Therapeutic drug monitoring of 5-fluorouracil. Cancer Chemother Pharmacol. 2016;78(3):447-64.
9. van Kuilenburg AB, Maring JG. Evaluation of 5-fluorouracil pharmacokinetic models and therapeutic drug monitoring in cancer patients. Pharmacogenomics. 2013;14(7):799-811.
10. Sougiannis AT, VanderVeen BN, Enos RT, Velazquez KT, Bader JE, Carson M, et al. Impact of 5 fluorouracil chemotherapy on gut inflammation, functional parameters, and gut microbiota. Brain Behav Immun. 2019;80:44-55.
11. Phillips E, France A, Thatvihane G, Nnaemeka U, Zaidi S. Mucositis and Cardiotoxicity Due to 5-Fluorouracil. Am J Ther. 2018;25(6):e712-e4.
12. Han Y, Yu Z, Wen S, Zhang B, Cao X, Wang X. Prognostic value of chemotherapy-induced neutropenia in early-stage breast cancer. Breast Cancer Res Treat. 2012;131(2):483-90.
13. Shitara K, Matsuo K, Takahari D, Yokota T, Inaba Y, Yamaura H, et al. Neutropaenia as a prognostic factor in metastatic colorectal cancer patients undergoing chemotherapy with first-line FOLFOX. Eur J Cancer. 2009;45(10):1757-63.
14. Kvinnsland S. The leucocyte nadir, a predictor of chemotherapy efficacy? Br J Cancer. 1999;80(11):1681.
15. Barreto R, Waning DL, Gao H, Liu Y, Zimmers TA, Bonetto A. Chemotherapy-related cachexia is associated with mitochondrial depletion and the activation of ERK1/2 and p38 MAPKs. Oncotarget. 2016;7(28):43442-60.
16. Williams GR, Deal AM, Shachar SS, Walko CM, Patel JN, O'Neil B, et al. The impact of skeletal muscle on the pharmacokinetics and toxicity of 5-fluorouracil in colorectal cancer. Cancer Chemother Pharmacol. 2018;81(2):413-7.
17. Botsen D, Ordan MA, Barbe C, Mazza C, Perrier M, Moreau J, et al. Dynapenia could predict chemotherapy-induced dose-limiting neurotoxicity in digestive cancer patients. BMC Cancer. 2018;18(1):955.
18. Sandini M, Patino M, Ferrone CR, Alvarez-Perez CA, Honselmann KC, Paiella S, et al. Association Between Changes in Body Composition and Neoadjuvant Treatment for Pancreatic Cancer. JAMA Surg. 2018;153(9):809-15.
19. Shitara K, Matsuo K, Oze I, Mizota A, Kondo C, Nomura M, et al. Meta-analysis of neutropenia or leukopenia as a prognostic factor in patients with malignant disease undergoing chemotherapy. Cancer Chemother Pharmacol. 2011;68(2):301-7.
20. Barreto R, Mandili G, Witzmann FA, Novelli F, Zimmers TA, Bonetto A. Cancer and Chemotherapy Contribute to Muscle Loss by Activating Common Signaling Pathways. Front Physiol. 2016;7:472.
21. Costamagna D, Costelli P, Sampaolesi M, Penna F. Role of Inflammation in Muscle Homeostasis and Myogenesis. Mediators of inflammation. 2015;2015:805172.
22. Sharma B, Dabur R. Role of Pro-inflammatory Cytokines in Regulation of Skeletal Muscle Metabolism: A Systematic Review. Current medicinal chemistry. 2020;27(13):2161-88.
23. Mahoney SE, Davis JM, Murphy EA, McClellan JL, Gordon B, Pena MM. Effects of 5-fluorouracil chemotherapy on fatigue: role of MCP-1. Brain Behav Immun. 2013;27(1):155-61.
24. Mahoney SE, Davis JM, Murphy EA, McClellan JL, Pena MM. Dietary quercetin reduces chemotherapy-induced fatigue in mice. Integr Cancer Ther. 2014;13(5):417-24.
25. Wang XS, Williams LA, Krishnan S, Liao Z, Liu P, Mao L, et al. Serum sTNF-R1, IL-6, and the development of fatigue in patients with gastrointestinal cancer undergoing chemoradiation therapy. Brain Behav Immun. 2012;26(5):699-705.
26. Abraham JE, Hiller L, Dorling L, Vallier AL, Dunn J, Bowden S, et al. A nested cohort study of 6,248 early breast cancer patients treated in neoadjuvant and adjuvant chemotherapy trials investigating the prognostic value of chemotherapy-related toxicities. BMC Med. 2015;13:306.
27. Yamanaka T, Matsumoto S, Teramukai S, Ishiwata R, Nagai Y, Fukushima M. Predictive value of chemotherapy-induced neutropenia for the efficacy of oral fluoropyrimidine S-1 in advanced gastric carcinoma. Br J Cancer. 2007;97(1):37-42.
28. Baechler S, Hobbs RF, Jacene HA, Bochud FO, Wahl RL, Sgouros G. Predicting hematologic toxicity in patients undergoing radioimmunotherapy with 90Y-ibritumomab tiuxetan or 131I-tositumomab. J Nucl Med. 2010;51(12):1878-84.
29. Tidball JG. Regulation of muscle growth and regeneration by the immune system. Nat Rev Immunol. 2017;17(3):165-78.
30. Mantovani A, Allavena P. The interaction of anticancer therapies with tumor-associated macrophages. J Exp Med. 2015;212(4):435-45.
31. Krippendorf BB, Riley DA. Distinguishing unloading- versus reloading-induced changes in rat soleus muscle. Muscle Nerve. 1993;16(1):99-108.
32. Liao X, Shen Y, Zhang R, Sugi K, Vasudevan NT, Alaiti MA, et al. Distinct roles of resident and nonresident macrophages in nonischemic cardiomyopathy. Proc Natl Acad Sci U S A. 2018;115(20):E4661-E9.
33. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29(6):313-26.
34. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol. 2014;14(8):571-8.
35. Frenette J, Chbinou N, Godbout C, Marsolais D, Frenette PS. Macrophages, not neutrophils, infiltrate skeletal muscle in mice deficient in P/E selectins after mechanical reloading. Am J Physiol Regul Integr Comp Physiol. 2003;285(4):R727-32.
36. Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J Exp Med. 2007;204(5):1057-69.
37. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 2005;5(12):953-64.
38. Reidy PT, McKenzie AI, Mahmassani ZS, Petrocelli JJ, Nelson DB, Lindsay CC, et al. Aging impairs mouse skeletal muscle macrophage polarization and muscle-specific abundance during recovery from disuse. Am J Physiol Endocrinol Metab. 2019;317(1):E85-E98.

39. Liu X, Liu Y, Zhao L, Zeng Z, Xiao W, Chen P. Macrophage depletion impairs skeletal muscle regeneration: The roles of regulatory factors for muscle regeneration. Cell Biol Int. 2017;41(3):228-38.
40. Farini A, Meregalli M, Belicchi M, Battistelli M, Parolini D, D'Antona G, et al. T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse. J Pathol. 2007;213(2):229-38.
41. Swamydas M, Lionakis MS. Isolation, purification and labeling of mouse bone marrow neutrophils for functional studies and adoptive transfer experiments. J Vis Exp. 2013(77):e50586.
42. St-Pierre J, Gregoire JC, Vaillancourt C. A simple method to assess group difference in RT-qPCR reference gene selection using GeNorm: The case of the placental sex. Sci Rep. 2017;7(1):16923.
43. Gilliam LA, Ferreira LF, Bruton JD, Moylan JS, Westerblad H, St Clair DK, et al. Doxorubicin acts through tumor necrosis factor receptor subtype 1 to cause dysfunction of murine skeletal muscle. J Appl Physiol (1985). 2009;107(6):1935-42.
44. Morton AB, Mor Huertas A, Hinkley JM, Ichinoseki-Sekine N, Christou DD, Smuder AJ. Mitochondrial accumulation of doxorubicin in cardiac and diaphragm muscle following exercise preconditioning. Mitochondrion. 2019;45:52-62.
45. Evans WJ, Morley JE, Argiles J, Bales C, Baracos V, Guttridge D, et al. Cachexia: a new definition. Clin Nutr. 2008;27(6):793-9.
46. Tarpey MD, Amorese AJ, Balestrieri NP, Fisher-Wellman KH, Spangenburg EE. Doxorubicin causes lesions in the electron transport system of skeletal muscle mitochondria that are associated with a loss of contractile function. J Biol Chem. 2019.
47. Barreto R, Kitase Y, Matsumoto T, Pin F, Colston KC, Couch KE, et al. ACVR2B/Fc counteracts chemotherapy-induced loss of muscle and bone mass. Sci Rep. 2017;7(1):14470.
48. Yi HJ, Hong KS, Moon N, Chung SS, Lee RA, Kim KH. Acute hyperammonemic encephalopathy after 5-fluorouracil based chemotherapy. Ann Surg Treat Res. 2016;90(3):179-82.
49. Liaw CC, Wang HM, Wang CH, Yang TS, Chen JS, Chang HK, et al. Risk of transient hyperammonemic encephalopathy in cancer patients who received continuous infusion of 5-fluorouracil with the complication of dehydration and infection. Anticancer Drugs. 1999;10(3):275-81.
50. Tidball JG. Inflammatory processes in muscle injury and repair. Am J Physiol Regul Integr Comp Physiol. 2005;288(2):R345-53.
51. Yang J, Zhang L, Yu C, Yang XF, Wang H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark Res. 2014;2(1):1.
52. Jablonski KA, Amici SA, Webb LM, Ruiz-Rosado Jde D, Popovich PG, Partida-Sanchez S, et al. Novel Markers to Delineate Murine M1 and M2 Macrophages. PLoS One. 2015;10(12):e0145342.
53. Huang SC, Wu JF, Saovieng S, Chien WH, Hsu MF, Li XF, et al. Doxorubicin inhibits muscle inflammation after eccentric exercise. J Cachexia Sarcopenia Muscle. 2017;8(2):277-84.
54. Guigni BA, Fix DK, Bivona JJ, 3rd, Palmer BM, Carson JA, Toth MJ. Electrical stimulation prevents doxorubicin-induced atrophy and mitochondrial loss in cultured myotubes. Am J Physiol Cell Physiol. 2019;317(6):C1213-C28.
55. Toth MJ, Voigt TB, Tourville TW, Prior SM, Guigni BA, Schlosberg AV, et al. Effect of neuromuscular electrical stimulation on skeletal muscle size and function in patients with breast cancer receiving chemotherapy. J Appl Physiol (1985). 2020;128(6):1654-65.
56. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 2014;6:13.
57. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. Nat Immunol. 2013;14(10):986-95.
58. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol. 2011;11(11):723-37.
59. Cote CH, Bouchard P, van Rooijen N, Marsolais D, Duchesne E. Monocyte depletion increases local proliferation of macrophage subsets after skeletal muscle injury. BMC Musculoskelet Disord. 2013;14:359.
60. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. J Cell Physiol. 2018;233(9):6425-40.
61. De Santa F, Vitiello L, Torcinaro A, Ferraro E. The Role of Metabolic Remodeling in Macrophage Polarization and Its Effect on Skeletal Muscle Regeneration. Antioxid Redox Signal. 2018.
62. Schiaffino S, Pereira MG, Ciciliot S, Rovere-Querini P. Regulatory T cells and skeletal muscle regeneration. FEBS J. 2017;284(4):517-24.
63. Zhang X, Chen Y, Hao L, Hou A, Chen X, Li Y, et al. Macrophages induce resistance to 5-fluorouracil chemotherapy in colorectal cancer through the release of putrescine. Cancer Lett. 2016;381(2):305-13.
64. Smuder AJ. Exercise stimulates beneficial adaptations to diminish doxorubicin-induced cellular toxicity. Am J Physiol Regul Integr Comp Physiol. 2019;317(5):R662-R72.
65. Johnson TA, Singla DK. PTEN inhibitor VO-OHpic attenuates inflammatory M1 macrophages and cardiac remodeling in doxorubicin-induced cardiomyopathy. Am J Physiol Heart Circ Physiol. 2018;315(5):H1236-H49.
66. Summan M, Warren GL, Mercer RR, Chapman R, Hulderman T, Van Rooijen N, et al. Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study. Am J Physiol Regul Integr Comp Physiol. 2006;290(6):R1488-95.
67. Segawa M, Fukada S, Yamamoto Y, Yahagi H, Kanematsu M, Sato M, et al. Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. Exp Cell Res. 2008;314(17):3232-44.
68. Zhao W, Lu H, Wang X, Ransohoff RM, Zhou L. CX3CR1 deficiency delays acute skeletal muscle injury repair by impairing macrophage functions. FASEB J. 2016;30(1):380-93.

69. Xiao W, Liu Y, Chen P. Macrophage Depletion Impairs Skeletal Muscle Regeneration: the Roles of Pro-fibrotic Factors, Inflammation, and Oxidative Stress. Inflammation. 2016;39(6):2016-28.
70. Inaba S, Hinohara A, Tachibana M, Tsujikawa K, Fukada SI. Muscle regeneration is disrupted by cancer cachexia without loss of muscle stem cell potential. PLoS One. 2018;13(10):e0205467.
71. Dunne RF, Loh KP, Williams GR, Jatoi A, Mustian KM, Mohile SG. Cachexia and Sarcopenia in Older Adults with Cancer: A Comprehensive Review. Cancers (Basel). 2019;11(12).
72. VanderVeen BN, Hardee JP, Fix DK, Carson JA. Skeletal muscle function during the progression of cancer cachexia in the male Apc(Min/+) mouse. J Appl Physiol (1985). 2018;124(3):684-95.
73. VanderVeen BN, Fix DK, Carson JA. Disrupted Skeletal Muscle Mitochondrial Dynamics, Mitophagy, and Biogenesis during Cancer Cachexia: A Role for Inflammation. Oxid Med Cell Longev. 2017;2017:3292087.
74. Carson JA BK. Interleukin-6 as a key regulator of muscle mass during cachexia. Exerc Sport Sci Rev. 2010;38(4):168-76.
75. VanderVeen BN, Fix DK, Montalvo RN, Counts BR, Smuder AJ, Murphy EA, et al. The regulation of skeletal muscle fatigability and mitochondrial function by chronically elevated interleukin-6. Exp Physiol. 2019;104(3):385-97.
76. Bonetto A AT, Jin X, Zhang Z, Zhan R, Puzis L, Koniaris LG, Zimmers TA. JAK/STAT3 pathway inhibition blocks skeletal muscle wasting downstream of IL-6 and in experimental cancer cachexia. American Journal of Physiology Endocrinology and Metabolism. 2012;303(3):E410-21.
77. Bonetto A AT, Kunzevitzky, Guttridge DC, Khuri S, Koniaris LG, Zimmers TA. STAT3 activation in skeletal muscle links muscle wasting and the acute phase response in cancer cachexia. PLoS One. 2011;6(7).
78. Shukla SK, Markov SD, Attri KS, Vernucci E, King RJ, Dasgupta A, et al. Macrophages potentiate STAT3 signaling in skeletal muscles and regulate pancreatic cancer cachexia. Cancer Lett. 2020;484:29-39.
79. Derman BA, Macklis JN, Azeem MS, Sayidine S, Basu S, Batus M, et al. Relationships between longitudinal neutrophil to lymphocyte ratios, body weight changes, and overall survival in patients with non-small cell lung cancer. BMC Cancer. 2017;17(1):141.

Tables

Table 1. Skeletal Muscle Immune Cell Population

		Total	Non-Debris	FSC Singlet	SSC Singlet	CD45+	CD45+ CD11b+	CD45+ CD11b+ F4/80+
Control	Mean	500000	357171	300554	293303	8313	6986	2509
	SEM	*0*	*(9278)*	*(12211)*	*(12898)*	*(1112)*	*(1092)*	*(531)*
5FU	Mean	500000	329763	265158	257197	4412*	3701*	1084*
	SEM	*0*	*(12996)*	*(15057)*	*(15433)*	*(397)*	*(384)*	*(182)*
p-value			0.207	0.180	0.171	0.005	0.009	0.013

Values are means ± SEM. Total number of cells counted. Absolute number of non-debris cells from the total number of cells. Absolute number of forward scatter (FSC) single cells from the non-debris cells. Absolute number of side scatter (SSC) single cells from the FSC single cells. Absolute number of CD45+ cells from the SSC single cells. Absolute number of CD45+CD11b+ cells from SSC single cells. Absolute number of CD45+CD11b+F4/80+ cells from SSC single cells. Significance was set at $p<0.05$. *Significantly different from Control using a student's t-test.

Table 2. Skeletal Muscle Activated Monocyte Population

		CD45+ CD11b+								
								F4/80+		
		Ly6c^{High}	Ly6c^{Low}	F480- Ly6c^{High}	F480+ Ly6c^{High}	F480+ Ly6c^{Low}	F480- Ly6c^{Low}	CD206- CD11c+	CD206+ CD11c+	CD206+ CD11c-
Control	Mean	1161	5824	685	475	1806	4018	852	385	646
	SEM	*(398)*	*(701)*	*(230)*	*(168)*	*(337)*	*(387)*	*(222)*	*(131)*	*(150)*
5FU	Mean	313*	3576*	119*	193	842*	2733*	250*	140*	423
	SEM	*(87)*	*(389)*	*(28)*	*(59)*	*(144)*	*(276)*	*(86)*	*(25)*	*(100)*
p-value		0.060	0.016	0.031	0.141	0.022	0.019	0.030	0.015	0.248

Values are means ± SEM. Absolute number of Ly6c^{High} cells from the CD45+CD11b+ single cells. Absolute number of Ly6c^{Low} cells from the CD45+CD11b+ s cells.Absolute number of F4/80-Ly6c^{High} cells from the CD45+CD11b+ single cells.Absolute number of F4/80+Ly6c^{High} cells from the CD45+CD11b+ single c number of F4/80+Ly6c^{Low} cells from the CD45+CD11b+ single cells. Absolute number of F4/80-Ly6c^{Low} cells from the CD45+CD11b+ single cells. Absolute n CD206-CD11c+ cells from the CD45+CD11b+F4/80+ single cells. Absolute number of CD206+CD11c+ cells from the CD45+CD11b+F4/80+ single cells. Abso CD206+CD11c- cells from the CD45+CD11b+F4/80+ single cells. Absolute number of CD206-CD11c- cells from the CD45+CD11b+F4/80+ single cells. Signifi at p<0.05. *Significantly different from Control using a student's t-test.

Table 3. Bone Marrow Immune Cell Population

		Total	Non-Debris	FSC Singlet	SSC Singlet	CD45+	CD45+ CD11b+	CD45+ CD11b+ Ly6c^{High}
Control	Mean	500000	483227	465831	464058	304878	233494	160170
	SEM	*0*	*(1160)*	*(2145)*	*(2295)*	*(50575)*	*(44979)*	*(40441)*
5FU	Mean	500000	409499*	401998*	400736*	246100	91678*	74938*
	SEM	*0*	*(8611)*	*(8845)*	*(8948)*	*(26514)*	*(19190)*	*(16942)*
p-value			0.00001	0.00003	0.00003	0.096	0.001	0.010

Values are means ± SEM. Total number of cells counted. Absolute number of non-debris cells from the total number of cells. Absolute number of forward scatter (FSC) single cells from the non-debris cells. Absolute number of side scatter (SSC) single cells from the FSC single cells. Absolute number of CD45+ cells from the SSC single cells. Absolute number of CD45+CD11b+ cells from SSC single cells. Absolute number of CD45+CD11b+Ly6c^{High} cells from SSC single cells. Significance was set at p<0.05. *Significantly different from Control using a student's t-test.

Figures

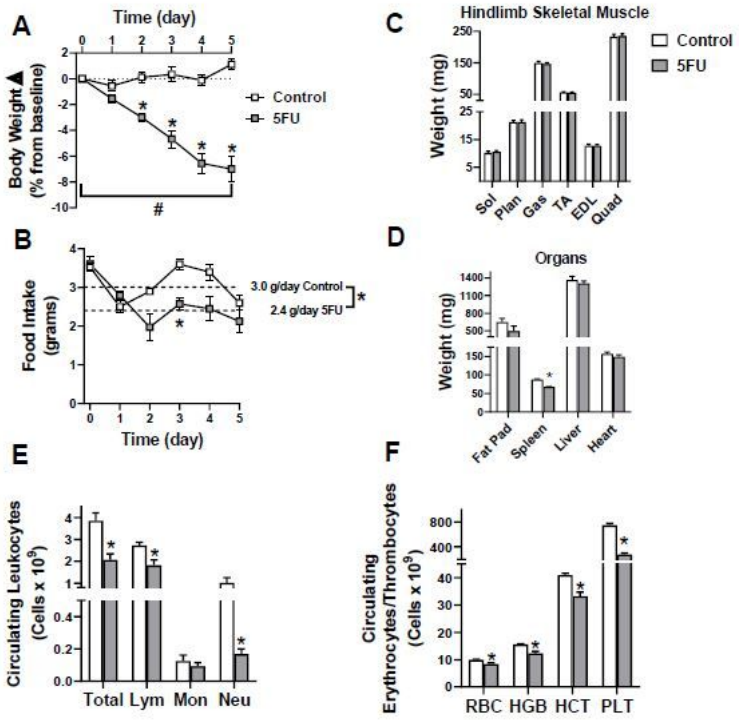

Figure 1

Animal characteristics. 5-fluorouracil (5FU) was solubilized in phosphate buffered saline at 3.5 mg/mL and administered to the mice at 35 mg/kg via intraperitoneal injection once daily for 5 days. A) Relative body weight change shown as the % change from day 0 throughout the duration of the study. B) Daily food intake in grams throughout the duration of the study. Dotted line illustrates the average daily food intake in grams (g) per day over the course of the 5 days of treatment. C) Select hindlimb muscle weights given in milligrams (mg) after 5 days of 5FU. D) Select organ weights in mgafter 5 days of 5FU. E)Circulating leukocytes given as # of cells x 109/L after 5 days of 5FU.F) Circulating erythrocytes and thrombocytes given as # of cells x 109/L after 5 days of 5FU. Soleus (Sol). Plantaris (Plan). Gastrocnemius (Gas). Tibialis anterior (TA). Extensor digitorum longus (EDL). Quadriceps (Quad). Lymphocytes (Lym). Monocytes (Mon). Neutrophils (Neu). Red blood cells (RBC). Hemoglobin (HBG). Hematocrit (HCT). Platelets (PLT). Significance was set at $p<0.05$. *Significantly different from Control using a student's t-test. #Significantly different from Day 0 using a repeated measures Two-way ANOVA.

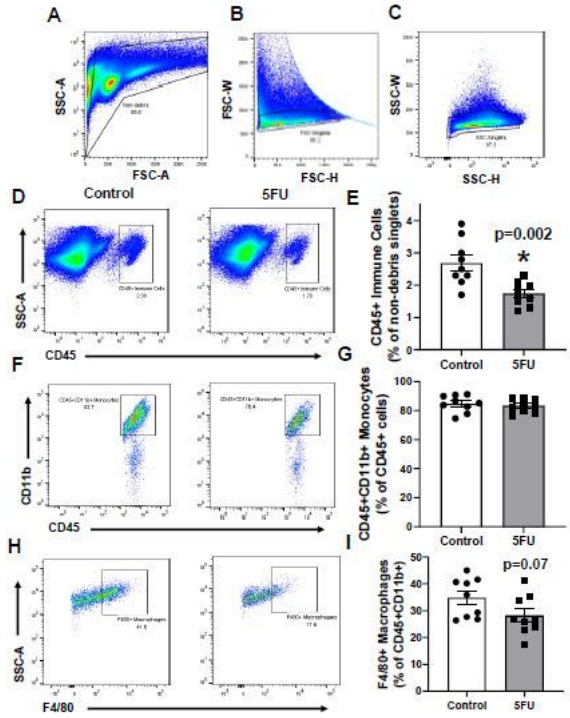

Figure 2

The effects of 5FU on skeletal muscle immune cells. A) Cells were gated for non-debris (SSC-A x FSC-A), B) FSC singlets (FSC-W x FSC-H), C) and SSC singlets (SSC-W x SSC-H; A-right).D) Non-debris singlet cells were then gated for total immune cells with CD45+. E) CD45+ cells were quantified and shown in the bar graph as the relative % of non-debris singlets. F) CD45+ cells were gated for monocytes with CD11b+.G) CD45+CD11b+ cells were quantified and shown in the bar graph as the relative % of CD45+ cells. H) CD45+CD11b+ were then gated for macrophages with F4/80. I) F4/80+ cells were quantified and shown in the bar graph as the relative % of CD45+CD11b+ cells. Significance was set at $p<0.05$. *Significantly different from Control using a student's t-test.

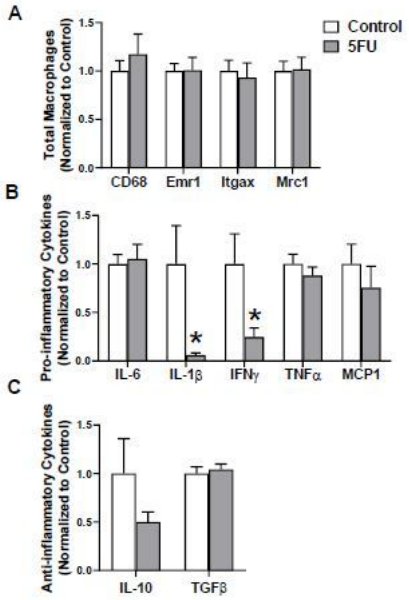

Figure 3

The effects of 5FU on skeletal muscle macrophage gene expression. A) Relative gene expression of total macrophage genes, CD68 and Emr1 (F4/80), M1-like macrophage gene, Itgax (CD11c), and M2-like macrophage gene, Mrc1 (CD206). B) Relative gene expression of pro-inflammatory genes, Interleukin (IL) 6, IL-1β,Interferon (IFN) γ, Tumor necrosis factor (TNF) α, and monocyte chemoattractant protein (MCP) 1. C) Relative gene expression of anti-inflammatory genes IL-10 and transforming growth factor (TGF) β. Significance was set at $p<0.05$. *Significantly different from Control using a student's t-test.

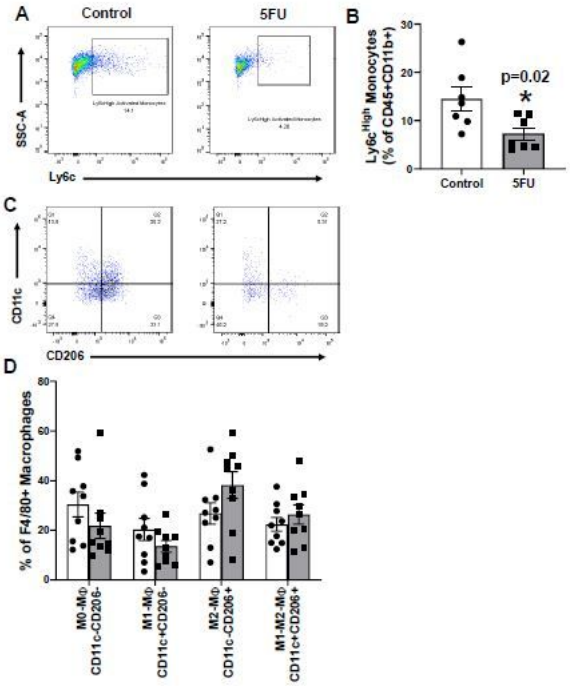

Figure 4

The effects of 5FU on infiltrating skeletal muscle monocytes and macrophages. A) CD11b+ monocytes were gated for their activation status using Ly6C. Cells were considered either resident (Ly6cLow) or activated/infiltrating (Ly6cHigh). B) Ly6cHigh monocytes were quantified and shown in the bar graph as relative

% of CD45+CD11b+ cells. C) F4/80+ macrophages were gated analyzed for their polarization status using CD11c and CD206. D) CD11c-CD206- cells were considered M0-like macrophages, CD11c+CD206- cells were considered M1-like macrophages,CD11c-CD206+ cells were considered M2-like macrophages, andCD11c+CD206+ cells were considered M1-M2-like transitional macrophages and graphed as the relative % of F480+ macrophages. Significance was set at p<0.05.*Significantly different from control (t-test).

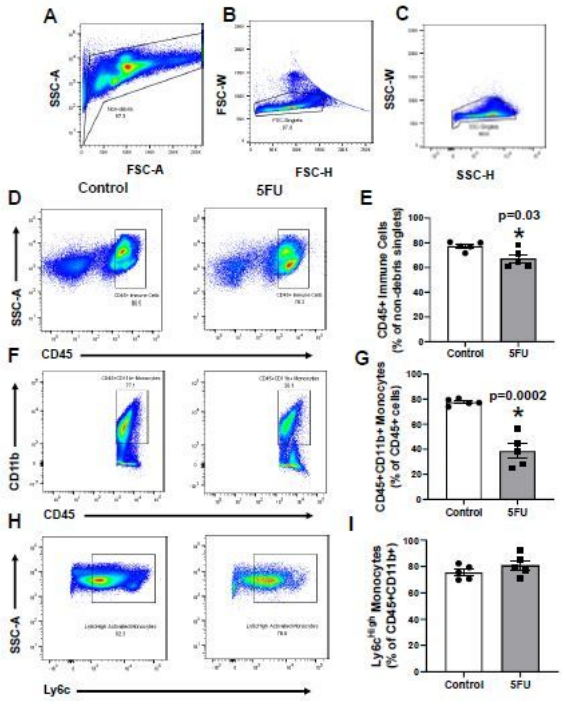

Figure 5

The effects of 5FU on bone marrow immune cells. A) Cells were gated for non-debris (SSC-A x FSC-A), B) FSC singlets (FSC-W x FSC-H), C) and SSC singlets (SSC-W x SSC-H; A-right).D) Non-debris singlet cells were then gated for total immune cells with CD45+. E) CD45+ cells were quantified and shown in the bar graph as the relative % of non-debris singlets. F) CD45+ cells were gated for monocytes with CD11b+.G) CD45+CD11b+ cells were quantified and shown in the bar graph as the relative % of CD45+ cells. H) CD11b+ monocytes were gated for their activation status using Ly6C. Cells were considered either resident (Ly6cLow) oractivated/infiltrating (Ly6cHigh). I) Ly6cHigh monocytes were quantified and shown in the bar graph as relative % of CD45+CD11b+ cells. Significance was set at p<0.05. *Significantly different from Control using a student's t-test.

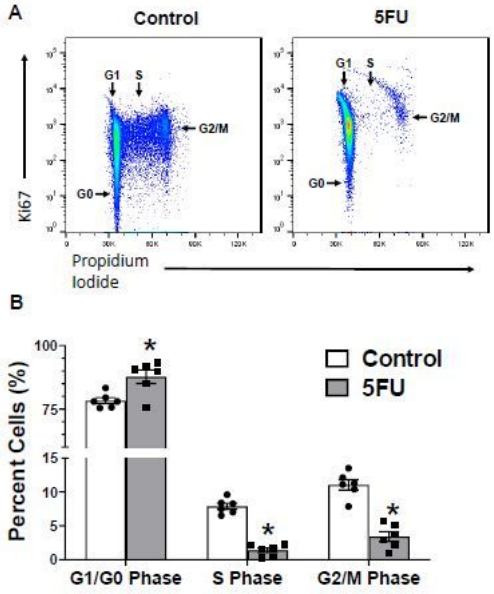

Figure 6

The effects of 5FU on bone marrow cell cycle. A) Cells were fixed and stained with Ki67 and propridium iodide (PI). B) Cells in the G1/G0, S, and G2/M phases were quantified and shown in the bar graph as therelative % of total cells. Significance was set at $p<0.05$.*Significantly different from control (t-test).