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The Effect of Altered Toll-like Receptor 4 Signaling on Cancer Cachexia

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Objective: To determine whether mice unable to mount an intact inflammatory response because of a Toll-like receptor (TLR) pathway defect will develop less severe cancer cachexia.

Design: Prospective animal study.

Setting: Academic research center.

Subjects: Six- to eight-week-old, female C3H/HeJ mice (17-18 g) and age-, weight-, and sex-matched wild-type C3H/HeN mice, differing in that the HeJ mice have nonfunctional TLR4 due to a TLR4 double mutation (TLR4d/d).

Intervention: The mice were inoculated with equal numbers of SCCF-VII cells and housed in individual cages.

Main Outcome Measures: Food intake, body weight, pretumor and posttumor body composition, circulating cytokines, and levels of a marker of muscle atrophy were analyzed.

Results: The wild-type HeN mice weighed less on average than the TLR4d/d mice (2.6 g vs 4.9 g) (P = .01). They consumed more food, had smaller tumors, and had less lean body mass and fat mass than the TLR4d/d mice. Interleukin 1β level was significantly elevated in the tumor-bearing HeN mice (mean gain of 259 pg/mL) but not in the TLR4d/d mice (P = .03). Both mouse strains had evidence of muscle atrophy.

Conclusions: In spite of increased food intake and smaller tumors, the wild-type HeN mice had more severe cachexia than the TLR4d/d mice. The impaired ability to secrete proinflammatory cytokines such as interleukin 1β may protect these animals from developing severe cancer cachexia. This animal model represents a novel system in which the host contributions to cachexia may be further studied.

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Cancer cachexia is a wasting syndrome that develops in the setting of advanced malignancy including squamous cell carcinoma of the head and neck. It results in reduced quality of life, decreased survival, and increased complications from treatment. Cachexia will be the main cause of death in approximately 20% to 30% of all patients with cancer. Clinically, cachexia manifests as unintentional weight loss, marked asthenia, sarcopenia, and anemia. Cachexia differs from starvation in that it is not reversible by increased nutritional intake, involves preferential loss of lean body mass rather than only fat mass, and is characterized by a chronic inflammatory state. Mounting evidence from animal models suggests a compelling link between activation of the inflammatory pathway and the development of cachexia. In addition to tumor-derived catabolic factors, humoral activators of the inflammatory cascade have been proposed as mediators of cancer cachexia. Interleukin 1β (IL-1β) and interleukin 6 (IL-6) are 2 proinflammatory cytokines that have been shown to stimulate the loss of lean body mass seen in experimental models of cancer cachexia. Interleukin 1β is necessary for the induction of IL-6–mediated cachexia in the colon-26 (C26) model, in which it also appears to contribute to the initiation of cachexia at the level of the tumor. Intratumoral injection of IL-1 receptor antagonist considerably attenuates weight loss and loss of lean body mass and fat mass.

Both sterile and infectious inflammation may be mediated by a common pathway involving similar proinflammatory cytokines. The Toll-like receptor (TLR) system is potentially one such pathway. Toll-like receptors are a family of innate cellular pathogen-recognition receptors that are present on many cell types including macrophages, dendritic cells, T and B cells,
tumor cells, and muscle cells.\(^8\) The classic ligand for TLR4 is lipopolysaccharide (LPS), commonly seen on gram-negative bacteria, but TLR4 is now known to recognize other endogenous ligands such as fibrinogen, heat-shock proteins, and polysaccharide fragments of heparin sulfate.\(^9\) Signaling through the TLR pathway not only results in activation of antigen-specific immunity but also leads to the production of the same cytokine mediators involved in infectious inflammation such as sepsis.\(^10\) Indeed, activation of the TLR4 receptor system by LPS elaborates the same proinflammatory cytokines that are known to mediate inflammation in cancer cachexia. One cytokine, IL-1\(\beta\), is capable of stimulating physiologic and muscle protein wasting in experimental models of cancer cachexia.\(^6\) Skeletal muscles, which express TLR2 and TLR4, respond to LPS and produce cytokines.\(^13,14\) Mice with this characteristic inflammation-induced wasting usually seen in response to tumor challenge using the SCCF-VII tumor cell line in both HeN (wild-type) mice and TLR4\(^{−/−}\) C3H/HeJ mice.

C3H/HeJ mice have a mutation in a single gene on chromosome 4, \(Ips\), which leaves the macrophages unable to respond to LPS and produce cytokines.\(^13,14\) Mice with this mutation in the TLR4 system (TLR4\(^{−/−}\) C3H/HeJ) experience impaired release of IL-1\(\beta\) and do not develop the characteristic inflammation-induced wasting usually seen in response to LPS challenge.\(^15-18\) Therefore, this strain of mice may represent an important model for the study of host cytokine contribution to the pathogenesis of cancer cachexia. In addition, host contribution to cancer cachexia may be studied in this model because mice with an impaired TLR4 signaling pathway appear to have different cytokine profiles than the cognic HeN mice with intact TLR signaling. Our hypothesis was that TLR4\(^{−/−}\) mice, with a defective TLR4 cytoplasmic domain, will have less severe cancer cachexia. In the present study, we compared the host response to tumor challenge using the SCCF-VII tumor cell line in both HeN (wild-type) mice and TLR4\(^{−/−}\) C3H/HeJ mice.

### METHODS

#### TUMOR CELL LINES

The SCCF cell line is a spontaneously arising squamous cell carcinoma that forms a measurable tumor after being injected into immunocompetent syngeneic mice. Cultured cells were propagated in tissue culture flasks at 37°C, with 5% carbon dioxide in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin sulfate, and 1% sodium glutamate. The SCCF cells were grown in culture to 80% confluence. On the day of tumor injection, cells were harvested using a short trypsin treatment (0.25% trypsin with 1.0-mmol/L EDTA) and then washed twice and resuspended in phosphate-buffered saline.

#### SERUM CYTOKINE AND CHEMOKINE ASSAYS

Cytokine and chemokine analysis was determined using Biorad Protein Array system (BioRad, Hercules, California), a novel multiplexed, particle-based, flow cytometric assay that uses anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of 2 fluorescent dyes. Our assay was customized to detect and quantify 18 cytokines and chemokines, including IL-1\(\beta\), IL-1\(\alpha\), IL-6, TNF-\(\alpha\), and interferon-\(\gamma\), among others. For each cytokine, 8 standards ranged from 2 to 32,000 pg/mL, and the minimum detectable dose was lower than 10 pg/mL. Prior to tumor inoculation and at the completion of the experiment, approximately 200 µL of blood was drawn. Gastrocnemius and quadriceps muscle, tumor, and epididymal fat were excised. Then, all tumors were excised and harvested the tumors at the completion of the study.

### ASSESSMENT OF FOOD INTAKE, BODY WEIGHT, AND TUMOR SIZE

The animals were individually housed for accurate measurement of food consumption. The amount of ingested food was measured in all cages every 2 days by taking the daily weights (grams) of food. Mean daily food intake and cumulative intake were determined for each group of mice. Body weight and the length (A) and width (B) of the tumors were measured every 2 to 4 days. Tumor growth was assessed using calipers in 2 dimensions. The mean orthogonal diameter of the tumor was determined using the following formula: mean diameter = \((A + B)/2\). The gross size of the tumors was determined after harvesting the tumors at the completion of the study.

### BODY COMPOSITION ANALYSIS

Prior to tumor injection, all animals underwent body composition analysis using a Lunar PIXImus densitometer 2.00 (software version 1.42.006.010; Lunar Corp., Madison, Wisconsin). The densitometer uses dual-energy x-ray absorptiometry to provide precise measures of fat mass, bone-free lean body mass, and total bone mineral density.\(^19\) The analysis was repeated on day 23 in live animals, prior to killing. Pretumor and posttumor results were compared in both groups.

### ANIMAL MODEL

Six- to eight-week-old, female TLR4\(^{−/−}\) C3H/HeJ mice (17-18 g) were purchased from Jackson Laboratories, Bar Harbor, Maine. Age-, weight-, and sex-matched C3H/HeN mice were purchased from Charles River Laboratories, Inc (Wilmington, Massachusetts). The animals were housed individually in sterilized plastic cages in a temperature-controlled room with a 12-hour dark-light cycle and were fed a defined AIN-93M diet (Granville Milling, Creedmoor, North Carolina) and received tap water ad libitum.

On day 0, the mice in each group received bilateral subcutaneous injections of the SCCF cells (1 × 10\(^6\) cells/100 µL). Food intake, body weight, and tumor size were measured every 1 to 2 days. On day 23, all mice were humanely killed and serum was drawn. Gastrocnemius and quadriceps muscle, tumor, and epididymal fat were excised. Then, all tumors were excised and final tumor weight was measured. Serum and tissues were snap frozen in liquid nitrogen and stored at −80°C. Final body weight was calculated by subtracting tumor weight from total body weight (weight of mice with tumors intact). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. Mice were allowed to grow tumors for 23 days or when the tumor burden had reached the accepted limit set by the Department of Laboratory Medicine and the IACUC. The animals were then humanely killed.
was collected from each mouse. Serum was harvested after the clotting of blood at room temperature for 1 hour and kept frozen (−80°C) until analysis. Pretumor and posttumor implantation cytokine levels were compared.

UBIQUITIN LIGASE EXPRESSION IN MUSCLES

On postimplantation day 23, the mice were humanely killed, and hind leg quadriceps and gastrocnemius muscles were rapidly dissected and snap-frozen in liquid nitrogen. Gastrocnemius and quadriceps muscles from tumor-bearing C3H/HeJ and C3H/HeN mice were homogenized. To confirm the presence of muscle atrophy, isolated muscle tissue was analyzed for the E3 ubiquitin ligase muscle ring finger 1 (MuRF1) using Northern blot analysis as previously described.20 Expression of MuRF1 was quantified by phosphoimaging and compared with the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene.

DATA ANALYSIS

All quantitative data are represented as mean±SE, with 95% confidence intervals (CIs) when appropriate. An unpaired t test allowing for unequal variance was used to compare group means. The reported P values, including those for cytokine analysis, are not corrected for multiple comparisons. Because of small sample sizes (5 mice per group), statistical testing results (P values) should be interpreted with care and analysis should be considered mainly as exploratory rather than inferential.

RESULTS

INCREASED FOOD INTAKE AND WEIGHT LOSS IN TUMOR-BEARING WILD-TYPE MICE

When equal numbers of SCCF cells were injected into the flanks of both strains of mice, the wild-type HeN mice weighed less than the TLR4<sup>−/−</sup> mice, even after tumor size was subtracted. The mean±SE weight gain in the HeN mice was 2.6±0.3 g (minus tumor), while the TLR4<sup>−/−</sup> mice gained 4.9±0.5 g (minus tumor) (P=.006) (Figure 1A). Beginning 1 week after tumor injection, the HeN mice stopped gaining weight. Despite the weight gain due to large tumors growing on the flanks, the wild-type mice started to lose weight beginning postinjection day 10 and had a significantly smaller change in body weight (15.7±1.9%) compared with the TLR4<sup>−/−</sup> mice (27.6±2.7%) (P=.01) (Figure 1B). The wild-type HeN mice without tumors usually have a mean±SE weight gain of 27%±7%, while the C3H/HeJ (TLR4<sup>−/−</sup>) mice without tumors have a weight gain of 18%±5% over the time course of these experiments. This suggests that the TLR4<sup>−/−</sup> strain of mice is less useful as a model for cancer cachexia because they do not exhibit the weight loss typically seen by the tumor-bearing C3H/HeN mice (Table 1).

The differences in body weight could not be attributed to anorexia or differences in food intake between the groups. The wild-type HeN mice consumed more food at every time point and had a mean±SE cumulative food intake of 102.5±5.8 g, while the TLR4<sup>−/−</sup> mice consumed 76.4±0.9 g (P=.002) (Figure 2A). In a separate experiment, HeN mice with and without tumors had their daily food intake monitored to rule out anorexia as a cause of the weight loss. The tumor-bearing mice consumed as much food as the controls (Figure 2B).

LESS TUMOR GROWTH IN WILD-TYPE MICE

The SCCF cell line is highly aggressive, with the formation of palpable tumors in mice by day 7 when injected in the flanks. Because of its reproducible rapid growth, tumors become so bulky that mice usually need to be killed...
by day 20 to 23. There was a significant difference in tumor size when measuring the mean tumor volume between mice with an intact TLR-4 pathway (C3H/HeN) and the TLR4d/d strain (Figure 3). The wild-type HeN mice had significantly smaller tumors, with a final mean±SE tumor volume of 1.35±0.3 cm² compared with TLR4d/d mice that had tumor volumes of 3.04±0.7 cm² (P = .05).

LESS LEAN BODY AND FAT MASS IN WILD-TYPE MICE

To determine whether the weight loss experienced by the mice was due to cachexia, each group of mice underwent pretumor and posttumor body composition analysis to examine the effect of SCCF tumor growth on lean body and fat mass. Body composition analysis revealed that tumor growth resulted in less lean body and fat mass in the wild-type mice than in the TLR4d/d strain (Figure 4). The wild-type HeN mice had a mean±SE percentage change in lean body mass of 27.5%±4.7%, while the TLR4d/d mice had a 56.5%±6.3% lean body mass change (P = .004). The percentage change in fat mass was also less in the HeN mice compared with the TLR4d/d mice, 17.8%±3.9% vs 34.6%±7.3% (P = .02). Figure 4 summarizes the results for the 2 groups of mice.

DIFFERENT CYTOKINE PROFILES IN THE 2 STRAINS OF MICE

There is evidence that activation of TLR4 system leads to the expression of some of the same proinflammatory cytokines involved in the production of cachexia. Cytokine production was therefore examined in the 2 congenic strains of mice bearing the same tumor type and was compared to see whether there were differences in the host’s ability to mount an inflammatory response. Of the 18 serum cytokine and chemokines analyzed, IL-1β was found to be significantly elevated in tumor-bearing HeN mice compared with the tumor-bearing TLR4d/d mice (P = .03) (Table 2 and Figure 5). Tumor necrosis factor α decreased in both strains of tumor-bearing mice (−242 vs −5053) (P = .34). Interferon-γ decreased in both strains as well (−1 vs −11) (P = .56).

MUSCLE WASTING IN BOTH STRAINS OF MICE

The E3 ubiquitin ligase MuRF1 targets proteins for degradation in the ubiquitin-proteosome system. Muscles undergoing protein degradation have elevated levels. When hind leg quadriceps muscles were analyzed for MuRF1, both strains of mice had evidence of muscle atrophy when compared with the control mice without tumors (Figure 6). The same regulation was seen with gastrocnemius muscles (data not shown).

COMMENT

The genetic predisposition of the host to mount an inflammatory response may determine the magnitude of cancer cachexia. Animal studies suggest that cytokine activation plays a central role in the genesis of fat and muscle catabolism seen in cachexia. In addition, tumor-
derived mediators such as proteolysis-inducing factor and lipid-mobilizing factor serve as a stimulus for proinflammatory cytokine production and contribute to the development of cachexia. Because LPS activation of the TLR4 pathway results in the production of the same inflammatory cytokines seen in cachexia, we hypothesized that the ability to activate the TLR pathway would lead to production of inflammatory mediators that may exacerbate cancer cachexia. Skeletal muscles express TLR4 and are known to mount an inflammatory response to LPS, with dose-dependent increases in muscle messenger RNA (mRNA) levels of IL-1β and other cytokines.11,12 Because cachexia is thought to be mediated by many of the same proinflammatory cytokines, it follows that TLR4<sup>−/−</sup> mice would develop less severe cancer cachexia.

In the present study, we demonstrate that TLR4<sup>−/−</sup> mice have evidence of muscle wasting but the cachexia was less severe than in their TLR4<sup>+/+</sup> counterparts. The wild-type mice had less fat and lean body mass, resulting in a significant difference in weight. This was also associated with a molecular marker of muscle atrophy, the up-regulation of the E3 ligase MuRF1, and elevated IL-1β levels.

To design effective therapeutic strategies to combat cachexia, a better understanding of the role of cytokines, both host and tumor derived, is essential. As such, we believe that C3H/HeM and C3H/HeJ (TLR4<sup>+/−</sup>) mice could serve as a model system to study altered host cytokine production. We tested the serum of wild-type and mutant mice for the presence of 18 cytokines and chemokines before and after tumor challenge. None of the TLR4<sup>−/−</sup> mice exhibited a significant intact inflammatory response after tumor challenge, which is consistent with results seen in experimental systems investigating the response to LPS-induced sepsis.11 The fact that these mice did not exhibit cachexia that was as severe supports the hypothesis that cytokine activation is important in the pathogenesis of cachexia.

To our knowledge, this model, HeN mice with SCCF tumor cells, is the first immunocompetent model of head and neck cancer cachexia. Previous work demonstrated that the SCCF tumor cells overexpress lipid-mobilizing factor, and this is associated with decreased fat mass in the tumor-bearing HeN mice.21 The addition of the C3H/HeJ (TLR4<sup>−/−</sup>) strain creates a novel system in which the host contributions to cachexia may be further studied. For instance, the role of TLR4 could be elucidated in this system, along with other downstream mediators of the TLR system, including nuclear factor-κB.

Several mechanisms have been proposed for the pathogenesis of wasting seen in cachexia, including the inappropriate production and release of cytokines, with or without activation of nuclear factor-κB. Nuclear factor-κB is a transcription factor that is activated by TNF-α, which reportedly inhibits skeletal muscle differentiation by suppressing myosin-D messenger RNA and protein. Reduced myosin-D expression impairs the repair of skeletal muscles, reducing lean body mass.22 Toll-like receptor 4 also induces activation of signaling molecules such as nuclear factor-κB.23 Because TLR4 is expressed on skeletal muscle cells and activation of TLR4 results in the activation of the same pathways seen in cytokine-induced protein degradation, we hypothesized that mice with intact TLR4 pathways would have more tumor-induced muscle wasting. Following tumor challenge, wild-type HeN mice exhibited the classic body composition changes seen in cachexia. Previous work demonstrated that hind leg weights are lower in wild-type mice with tumors than in control mice.21 Body composition analysis confirmed that wild-type mice exhibited less lean body and fat mass compared with TLR4<sup>−/−</sup> mice. This was manifested as a significant difference in weight despite increased food consumption.

Although we have provided preliminary evidence that TLR4 may be a key determinant of the severity of tumor-induced cachexia, more research is needed to determine the exact mechanism by which this pathway contributes to tumor-associated inflammation and wasting. It is possible that IL-1β mediates muscle wasting through the inhibition of protein synthesis and the not the proteasome pathway because both strains of mice had up-regulation of MuRF1, yet the TLR4<sup>−/−</sup> mice had more lean body mass. In addition, the matched TLR4<sup>−/−</sup> mice had significantly larger tumors, which merits further investigation. This may demonstrate that targeted therapies for cancer cachexia may not necessarily be sound anticancer therapies. Therefore,
careful studies of TLR antagonists and antiinflammatory agents will have to be performed.

In conclusion, in spite of increased food intake and smaller tumors, the mice with an intact TLR4 signaling pathway experienced more severe cachexia than the mice with deficient signaling. This was evidenced by lower body weights, less lean body mass and fat mass, and clinical evidence of wasting compared with the age- and weight-matched TLR4d/d mice. These results are more striking because wild-type mice without tumors tend to gain slightly more weight than the TLR4d/d mice. Interleukin 1β appears to be an important mediator of cachexia in this model. Since the mice are cogenic except for this double mutation in TLR4 pathway signaling and are inoculated with the same number of identical tumor cells, the impaired ability to secrete proinflammatory cytokines such as IL-1β may protect these animals from de-

### Table 2. Comparison of Pretumor and Posttumor Serum Cytokine and Chemokine Analyses

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>C3H/HeN Mice, Mean (Posttumor – Pretumor Level)</th>
<th>C3H/HeJ Mice, Mean (Posttumor – Pretumor Level)</th>
<th>Mean (95% CI) Difference of C3H/HeN – C3H/HeJ Levels</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>259</td>
<td>-100</td>
<td>359 (50 to 668)</td>
<td>.03</td>
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<tr>
<td>IFN-γ</td>
<td>-1</td>
<td>-11</td>
<td>10 (30 to 51)</td>
<td>.56</td>
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<tr>
<td>IL-6</td>
<td>27</td>
<td>-112</td>
<td>139 (198 to 477)</td>
<td>.33</td>
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<tr>
<td>TNFα</td>
<td>-242</td>
<td>-5053</td>
<td>4811 (-7517 to 17138)</td>
<td>.34</td>
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<tr>
<td>IL-1α</td>
<td>-76</td>
<td>-224</td>
<td>148 (-204 to 501)</td>
<td>.32</td>
</tr>
<tr>
<td>IL-2</td>
<td>0</td>
<td>-12</td>
<td>18 (-1 to 37)</td>
<td>.06</td>
</tr>
<tr>
<td>IL-4</td>
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<td>-1</td>
<td>1 (-1 to 2)</td>
<td>.22</td>
</tr>
<tr>
<td>IL-5</td>
<td>-3</td>
<td>-4</td>
<td>1 (-4 to 7)</td>
<td>.61</td>
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<tr>
<td>IL-10</td>
<td>89</td>
<td>-3</td>
<td>92 (-194 to 379)</td>
<td>.41</td>
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<tr>
<td>GM-CSF</td>
<td>5</td>
<td>-3</td>
<td>8 (-16 to 30)</td>
<td>.42</td>
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<tr>
<td>IL-3</td>
<td>-2</td>
<td>-1</td>
<td>-1 (-8 to 5)</td>
<td>.60</td>
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<td>IL-12 (p40)</td>
<td>44</td>
<td>193</td>
<td>148 (-937 to 641)</td>
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<tr>
<td>IL-12 (p70)</td>
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<td>-83</td>
<td>153 (-171 to 478)</td>
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<td>IL-17</td>
<td>129</td>
<td>-160</td>
<td>289 (-253 to 832)</td>
<td>.24</td>
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<tr>
<td>G-CSF</td>
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<td>-136 (-544 to 271)</td>
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<td>KC</td>
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<td>230</td>
<td>-132 (-413 to 150)</td>
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<tr>
<td>MIP-1α</td>
<td>20</td>
<td>-15</td>
<td>35 (-4 to 74)</td>
<td>.07</td>
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<tr>
<td>RANTES</td>
<td>143</td>
<td>233</td>
<td>-90 (-294 to 114)</td>
<td>.32</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; GM-CSF, granulocyte macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; IFN-γ, interferon γ; IL, interleukin; TNFα, tumor necrosis factor α; KC, mouse chemokine N51; MIP-1α, macrophage inflammatory protein 1α; RANTES, regulated on activation, normal T expressed and secreted.

a Unless otherwise indicated, results are expressed as picogram per milliliter concentrations of the differences between the pretumor and posttumor levels. Serum collected from wild-type C3H/HeN and TLR4d/d C3H/HeJ mice was analyzed for 18 different cytokines using the Bioplex protein array system (Biorad, Hercules, California). Only IL-1β was found to be significantly elevated following tumor challenge.
veloping more severe cancer cachexia. This animal model represents a novel system in which host contributions to cachexia may be further studied.

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Author Contributions: Dr Couch had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Cannon, Guttridge, Dahlman, Lai, and Couch. Acquisition of data: Cannon, Guttridge, Dahlman, Lai, and Couch. Analysis and interpretation of data: Cannon, George, Bůžkova, and Couch. Critical revision of the manuscript for important intellectual content: Cannon, Dahlman, Lai, Shores, and Couch. Statistical analysis: Bůžkova and Couch. Obtained funding: Couch. Administrative, technical, and material support: Guttridge, Dahlman, George, Lai, and Couch. Study supervision: Couch.

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