

# Tumor necrosis factor signaling is implicated in intestinal mucosal injury of cancer cachexic mice

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**Abstract:** This study will examine the relationship between tumor necrosis factor (TNF) and intestinal mucosal injury in a cancer cachexia mouse model. The C26 adenocarcinoma cancer model was set up, and immunohistochemistry was performed to investigate the histological changes of injuring the intestinal mucosa and the intestinal villi. The liquid-phase protein chip was employed to examine the changes in TNF and its receptors. The results demonstrated an intestinal mucosal injury in the cancer cachexia mouse model. The height of the intestinal villi decreased, and thinner basal membrane thickness was noted. The serum TNF-alpha and sTNFR1 increased, while in the mucosa, the TNF-alpha concentration increased; sTNFR1 and sTNFR2 decreased when compared to the control group. In conclusion, there is a potential association between the TNF signaling pathway and the intestinal mucosal injury in a cancer cachexia mouse model. Such understanding provides insights into the development of novel therapeutic targets for intestinal mucosa protection in clinical practice.

**Keywords:** tumor necrosis factor, cachexia model, inflammation, proinflammatory factors, injury

## Introduction

The integrity of the intestinal mucosal barrier is essential for cachexic patients with complicating cancerous malnutrition. Intestinal mucosal injury not only affects the absorption and utilization of nutrients, but it can also lead to bacterial translocation and gut-derived sepsis.<sup>1</sup> The mechanisms involved in intestinal mucosal injury are complex.<sup>1,2</sup> The gastrointestinal tract is a target for inflammatory responses, and the inflammatory response is likely to be implicated in intestinal mucosal injury. Cytokine alterations in patients with cancerous malnutrition may adversely impact the patients' metabolism and immune function. The underlying cytokines that are potentially involved in the injury of the cachexic mucosal barrier consist of two classifications, namely proinflammatory factors, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, IFN-gamma, and leukemia inhibitory factor (LIF), as well as anti-inflammatory factors, such as sTNFR, IL-1receptor antagonists, IL-4, IL-10, IL-13, and IL-15.<sup>2</sup>

In addition to endotoxins, long-term starvation and certain physiochemical factors are generally accepted as the contributing factors of intestinal mucosal damages. It is known that TNF alters aberrantly in cancer cachexia patients;<sup>2</sup> however, it remains unknown whether TNF signaling is implicated in intestinal mucosal injury in these patients. The primary objective of this study is to characterize the TNF signaling profile in the circulation and intestinal tissues in a cancer cachexia mouse model with histological evidence of intestinal barrier impairment.

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## Materials and methods

### Animals and cancer cachexia model

The use and care of animals was compliant with the institutional regulations established and approved by the Animal Research Committee at Jinling Hospital, Nanjing, China. Specific-pathogen-free inbred male BALB/c mice aged 6–8 weeks, and weighing 20–24 g were bred and housed in the Laboratory Animal Center at Jinling Hospital. Animals were given free access to rodent chow containing 20%–25% protein, 5%–10% fat, and 3%–5% crude fiber, as well tap water, ad libitum. Mouse colon 26 adenocarcinoma cell line was purchased from the Institute of Materia Medica (Chinese Academy of Medical Sciences, Beijing, China). Tumor cells were propagated in BALB/c mice for three to four passages through small volume implantation.<sup>1</sup> The tumors that were free of connective tissues and necrotic tissues were homogenized in 2 mL/g of normal saline. One million tumor cells were injected into the axillary fossa of mice (cachexia group,  $n = 7$ ) at a volume of 0.1 mL in order to establish the cancer cachexia model, while normal saline was injected using the same protocol in the control group ( $n = 7$ ).

### Histological validation of intestinal mucosal injury

All the mice were sacrificed 16 days following injection. A longitudinal abdominal incision was made to harvest the small intestine from the Trietz ligament. The small intestine was fashioned into 1 cm segments and fixed in 10% formaldehyde at room temperature for 24 hours. The intestinal segments were embedded in paraffin and were consecutively cut into 5  $\mu$ m sections on a microtome. The intestinal sections were stained with hematoxylin-eosin, and they were assessed for the height and width of small intestinal villi, the thickness of the intestinal muscular layer, and the thickness of the mucosal basal layer, which were measured using a pathological image analysis system (Olympus Corporation, Tokyo, Japan).

### Enzyme-linked immunoabsorbent assay of serum TNF

Venous blood samples were collected from the mice prior to being sacrificed, via the angular vein. The blood samples were maintained static at 4°C for 24 hours to separate the serum, and the serum was cryopreserved at –70°C for further experiments. Serum TNF- $\alpha$  was detected using

the Luminex liquid protein microarray detection system (Life Technologies Corporation, Carlsbad, CA), and soluble TNF receptors 1 and 2 (sTNFR1/2) were determined using an ELISA kit (Life Technologies Corporation), as per the manufacturer's instructions.

### Enzyme-linked immunoabsorbent assay of intestinal TNF

Following the harvest of the small intestines, the mesenteries were torn off, and the distal end of the small intestine was ligated using a silk suture. The intestinal segment was flip-flopped using a tenacious blunt guidewire, which faced the mucosa outwards. The intestinal lumen was irrigated with normal saline. Small intestinal specimens (approximately 400 g) were fashioned into 1 cm segments, homogenized in a glass homogenizer, and centrifuged at 10,000 rpm at 4°C for 30 minutes. The supernatants were collected and stored at –70°C for further use. Intestinal TNF- $\alpha$  was detected using the Luminex liquid protein microarray detection system (Life Technologies Corporation), and sTNFR1/2 levels were determined using the aforementioned ELISA kit.

### RNA extraction

Following the rinse in normal saline, the mucosal layer was sheared off the small intestine using a surgical scalpel in a longitudinal direction. The mucosal specimens were homogenized by pipetting, and the mucosal cells were resuspended, washed, and centrifuged in normal saline at 1800 g at 4°C for 5 minutes. The cell suspension was supplemented with the Trizol reagent (Qiagen, Valencia, CA) at a ratio of 1 mL per 100 mg cells, and stored at –70°C for further experiments. RNA extraction was performed as per the manufacturer's instructions. Briefly, chloroform was added into TRIzol<sup>®</sup>-treated cells (Life Technologies Corporation) at a ratio of 1:5 (v/v), followed by a 3-minute incubation period at room temperature, and 15 minutes of centrifugation at 12,000 g and at 4°C. The supernatants were collected and transferred into an Eppendorf tube, and isopropanol was supplemented at a ratio of 0.6:1 (v/v), allowing for incubation at room temperature for 10 minutes. Following a centrifugation at 12,000 g at 4°C for 10 minutes, the supernatant was discarded, and the RNA pellet was dissolved in 75% (v/v) ethanol. The pellet was then centrifuged at 7500 g at 4°C for 5 minutes, and was left to air-dry at room temperature for 5–10 minutes, and dissolved in RNase-free water (Promega Corporation, Southampton, UK) for further reverse transcription.

## Reverse transcription polymerase chain reaction

DNase 1 (1 u/μL; Promega Corporation, Madison, WI) was added to RNA samples at a ratio of 1:8 (v/v) to digest genomic DNA at room temperature for 15 minutes. DNase 1 was inactivated by the incubation with 25 mM EDTA at 65°C for 15 minutes. The concentration and purity of RNA samples were determined using an ultraviolet spectrophotometer. RNA was pretreated in a cocktail solution containing 4 μg of RNA, 20 mM of glyceraldehyde phosphate dehydrogenase (GAPDH), and 1 μg of antisense primers (Table 1) at 70°C for 5 minutes, and this solution was subsequently chill shocked in an ice bath. RNA was reversely transcribed to cDNA in a 25 μL mixture containing 1.5 μL of 10 mM of dNTP, a 25-unit RNase inhibitor, a 200-unit M-MLV (Promega Corporation, Madison, WI), and RNase-free water at 42°C for 60 minutes. The polymerase chain reaction (PCR) system (50 μL) consisted of 3 μL of cDNA, 1 μL of 20 mM sense and antisense primers, 1 μL of 10 mM dNTP, 5 μL of 25 mM MgCl<sub>2</sub>, 5 μL of 10 × buffer, and a 2.5-unit Taq enzyme (Jingmei Co, Ltd, Beijing, China). The thermal cycling conditions were as follows: initiated at 95°C for 5 minutes; denatured at 95°C for 5 minutes; annealed at 54°C for GAPDH, at 58°C for TNF, or at 56°C for TNFR-DD to 7258°C for TNF for 1 minute and for 35 cycles; and elongated at 72°C for 10 minutes. The PCR products were electrophoresed on 1.5% agarose gel and visualized using a gel imaging system (Shanghai Forte Land Co, Ltd, Shanghai, China).

## Statistical analysis

The Statistical Package for the Social Sciences 16.0 software (SPSS, Chicago, IL) was used for statistical analyses. All the data were expressed as the mean ± SD, and the intergroup difference was compared using the Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

## Results

### Cancer cachexia mouse model

The initial body weight was comparable between the two groups (cachexia versus controls, 21.7 ± 1.8 g versus

21.9 ± 1.8 g, *P* > 0.05, respectively). The axillary tumor became palpable in the experiment group 5 days following the injection, whilst the symptoms suggestive of cachexia emerged 8–9 days following inoculation. The tumor-bearing mice started to experience rapid weight loss when the tumor experienced growth to 1 cm<sup>3</sup>. On day 16 of inoculation, the mean nontumor body weight of the experimental group approximated 62% of that of the control group (15.6 ± 1.6 g versus 25.4 ± 1.4 g, *P* < 0.01, respectively) (Figure 1).

### Intestinal mucosal injury in cancer cachexic mouse

On intestinal mucosal histology, the width of the intestinal villi and the thickness of the muscular layer were comparable between the cachexia and the control groups, respectively (villus width, 29.7 ± 4.3 μm versus 37.0 ± 5.1 μm, *P* > 0.05; muscular layer thickness, 21.0 ± 5.6 μm versus 19.6 ± 5.3 μm, *P* > 0.05). However, the height of the villi and the thickness of the basal layer were significantly less in the cachexia group than in the control group (villus height, 93.2 ± 8.3 μm in the cachexia group versus 222.7 ± 43.5 μm in the control group, *P* < 0.01; basal layer thickness, 48.3 ± 4.9 μm in the cachexia group versus 52.3 ± 7.3 μm in the control group, *P* < 0.05, respectively) (Figure 2).

### Serum TNF-alpha, sTNFR I, and sTNFR2

The cancer cachexic mice exhibited a significantly higher serum TNF-alpha (19.4 ± 7.1 pg/mL) and sTNFR1 (1349.6 ± 367.0 pg/mL) than the control mice (TNF-alpha: 11.4 ± 6.3 pg/mL, *P* < 0.05; sTNFR1: 675.8 ± 40.5 pg/mL, *P* < 0.01). However, serum sTNFR2 remained comparable between the two groups (142.9 ± 4.4 pg/mL for cachexic mice versus 181.7 ± 32.2 pg/mL for controls, *P* > 0.05) (Figure 3).

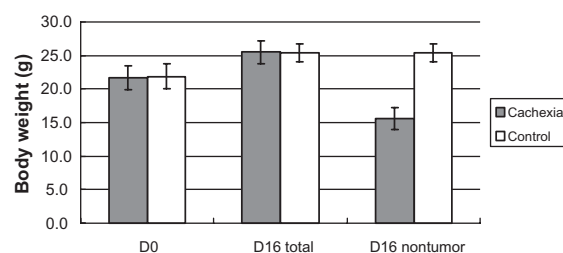
### Intestinal TNF-alpha, sTNFR I, and sTNFR2

The cancer cachexic mice exhibited a significantly higher intestinal TNF-alpha (2.9 ± 0.8 pg/mL) than the control mice

**Table 1** Primer pairs for reverse transcription PCR

Gene	Primer sequence (5' to 3')		Size of PCR product (bp)
	Sense	Anti-sense	
TNF-alpha	AACTAGTGGTGCCAGCCGAT	CTTCACAGAGCAATGACTCC-3	334
TNFR-DD	CAGGCACGAGAACGGCATAG	GGCACACTGTATGGGGAGC	161
GAPDH	CTGGTGCTGAGTATGTCGTG	CAGTCTTCTGAGTGCCAGTG	294

**Abbreviations:** bp, base pair; DD, death domain; PCR, polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.



**Figure 1** Body weight chart of cachexia versus control mice.

**Note:**  $P < 0.01$  for nontumor body weight on day 16 of inoculation.

( $0.3 \pm 0.1$  pg/mL,  $P < 0.05$ ). However, the concentrations of sTNFR1 and sTNFR2 were significantly lower in the cachexia group than in the control group (sTNFR1,  $13.6 \pm 1.1$  pg/mL in the cachexia group versus  $54.3 \pm 5.8$  pg/mL in the control group,  $P < 0.01$ ; sTNFR2,  $1.3 \pm 0.4$  pg/mL in the cachexia group versus  $3.9 \pm 0.8$  pg/mL in the control group,  $P < 0.001$ ) (Figure 4).

## Intestinal mucosal expression of TNF-alpha and TNFR-DD genes

TNF-alpha expression was detected in the mucosal cells of cachexic mice at a ratio intensity of  $0.61 \pm 0.04$  (TNF-alpha/GAPDH), whereas TNF-alpha expression was undetectable in the mucosal cells of control mice. However, the expression intensity of TNFR-DD was comparable between the two groups (cachexia  $1.10 \pm 0.06$  versus control  $1.12 \pm 0.02$ ,  $P > 0.05$ ) (Figure 5).

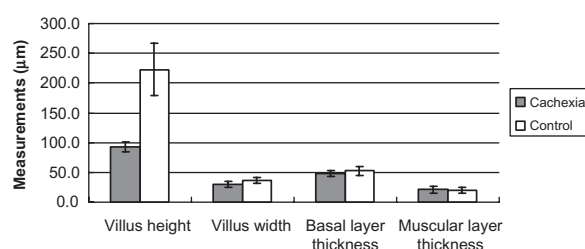
## Discussion

Cancerous malnutrition is a syndrome characterized by anorexia, weight loss, infirmness, and anemia. More than 80% of cancer patients are complicated with malnutrition, and 22% of them die of cachexic malnutrition.<sup>1</sup> It is generally accepted that systemic inflammatory responses underlie patients with complicating cancer cachexia. The gastrointestinal tract is a target organ of inflammatory response, and the inflammatory response is likely to be implicated in

intestinal mucosal injury. Cytokine alterations in patients with cancerous malnutrition may adversely impact patients' metabolism and immune function. The underlying cytokines that are potentially involved in the cachexic mucosal barrier injury consist of two classifications, namely proinflammatory factors, such as TNF, IL-1, IL-6, IFN-gamma and LIF, and anti-inflammatory factors, such as sTNFR, IL-1 receptor antagonist, IL-4, IL-10, IL-13, and IL-15.<sup>2</sup>

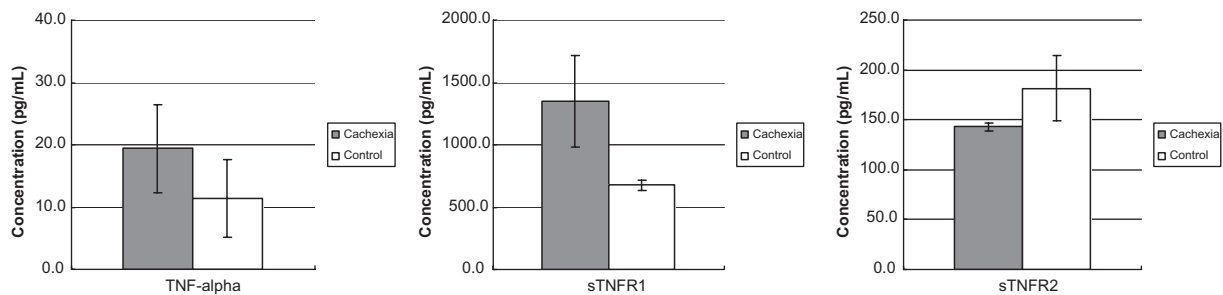
Among these cytokines, TNF plays a key role in inflammatory responses. TNF is mainly produced in the Paneth cells that are located in the lamina propria of the small intestine. Under inflammatory infiltration conditions, monocytes, macrophages, and eosinophils can also secrete TNF.<sup>9,10</sup> TNF-alpha exerts biological effects on its receptors through endocrine and paracrine mechanisms. Previous animal experiments have shown that the inoculation site of tumor cells may be associated with the occurrence of anorexia and weight loss.<sup>3,4</sup> Animals whose brains bear TNF-alpha-secreting tumors exhibit rapid anorexia and weight loss, whereas those muscle-tumor-bearing animals have been shown to progress to cachexia in the absence of preceding anorexia.<sup>5</sup> The injection of TNF-alpha also increases the circulating IL-1 level, while the increased IL-1 inhibits the hypothalamic feeding center and suppresses the gastrointestinal emptying function.<sup>6-8</sup>

When the mucosa of the small intestine are subjected to endotoxins, the expression of TNF is significantly upregulated in the intestinal tissues.<sup>10,11</sup> The high expression of TNF has been documented in patients with gastrointestinal malignancies.<sup>12</sup> TNF increases the permeability of the intestinal mucosal barrier through the destruction of the epithelial tight junction, as well as by inducing the apoptosis of epithelial cells. An experiment on intestinal mucosal gas conduction in an animal model of inflammatory bowel disease has shown that the increased intestinal mucosal permeability is derived from epithelial apoptosis (56%) and tight junction impairment (44%). The binding of TNF to its receptors activates the I $\kappa$ B/NF $\kappa$ B signaling pathways, causing the release of inflammatory cytokines,<sup>13,14</sup> thus impairing the tight junction of intestinal mucosal cells.<sup>15</sup> Additionally, TNF could directly induce the upregulation of caspases 1 and 3 in the presence of TNFR-DD; moreover, it could also lead to the apoptosis of endothelial cells,<sup>16-18</sup> and promote the production of matrix metalloproteinases by mesenchymal cells.<sup>19</sup> These effects disturbed the microcirculation of the intestinal mucosa, damaged the oxygen transportation to the epithelial cells by lipid peroxidation, and gave rise to impairments in the intestinal mucosal barrier. The mucosal barrier injury



**Figure 2** Intestinal mucosal measurements of cachexia mice ( $n = 7$ ) versus control mice ( $n = 7$ ) on hematoxylin-eosin staining at day 16 following the inoculation.

**Notes:**  $P < 0.01$  for villus height, and  $P < 0.05$  for basal layer thickness.



**Figure 3** Serum TNF-alpha, sTNFR1, and sTNFR2.

**Notes:**  $P < 0.05$  for TNF-alpha;  $P < 0.01$  for sTNFR1.

**Abbreviations:** TNF, tumor necrosis factor; sTNFR, soluble tumor necrosis factor receptor.

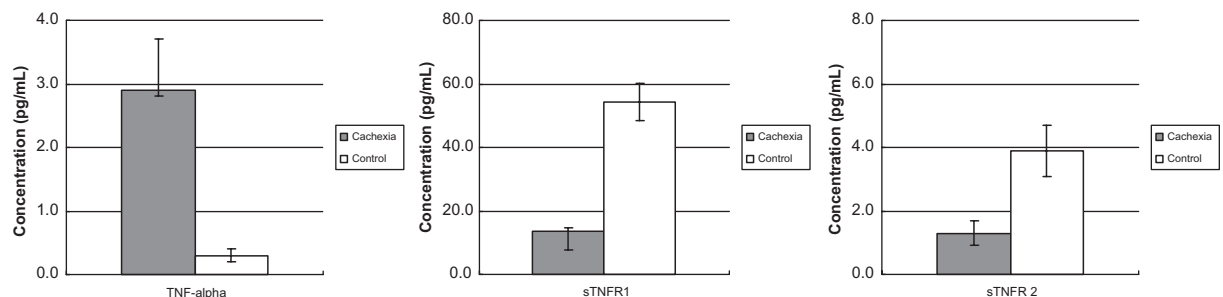
manifests mainly as intestinal mucosal edema, atrophy of intestinal villi, hyperplasia of crypt cells, loss of goblet cells, and hypersecretion of mucin.<sup>20</sup> The impaired intestinal mucosal barrier is prone to the translocation of bacteria and endotoxins. An in vitro study has shown that the decrease of TNF-alpha by neutralization can improve the permeability of the intestinal mucosal barrier.<sup>21</sup> The inhibition of the caspase signaling pathway can also suppress TNF-induced epithelial cell apoptosis.<sup>2</sup>

The findings from our animal study have shown that cancer cachexic mice have shorter villi and a thinner mucosal basal layer, indicating that there is atrophy of intestinal mucosa in cancerous malnutrition that is similar to that in inflammatory bowel disease. The elevation in serum TNF-alpha and TNFR1 is suggestive of the presence of systemic inflammatory responses in cancer cachexia. Moreover, the secretion of TNF-alpha is increased in the intestinal tissues of cachexic mice with complicating mucosal barrier injuries, whereas the secretion of sTNFR1/2 is decreased in the case of mucosal barrier impairment. This indicates that sTNFR exerts a protective effect on the intestinal mucosal barrier. The PCR results further confirm that the expression of the TNF-alpha gene is highly upregulated in the mucosal cells of cachexic mice, whilst that of TNFR-DD remains unchanged.

Lymphocytes residing in the intestinal mucosa may cause the overexpression of TNF-alpha in the context of cancerous malnutrition, which breaks down the tight junction of intestinal epithelial cells and impairs the intestinal mucosal barrier.

The production and release of cytokines are regulated by multiple factors, including the cytokine-cytokine interaction, the underlying disease process, the site of the disease, and the property of the pathogen. Our results show that serum TNF levels do not parallel intestinal TNF level in cachexic mice as compared to control mice. This variation in TNF levels may be attributed to the variable production of TNF by tumor cells, and it may also result in response to the contribution of pathogenic bacteria. Gram-negative bacteria primarily stimulate macrophages to produce IL-1 and IL-6, whereas these bacteria do not significantly alter TNF secretion.<sup>22</sup> In contrast, staphylococcal enterotoxin B increases the intestinal mucosal permeability, in which TNF plays a primary role.<sup>23</sup>

In conclusion, TNF signaling is implicated in intestinal mucosal barrier impairment. The increase in serum and intestinal TNF as well as the decrease in serum sTNFR is associated with intestinal mucosal injury in a cancer cachexic mouse model.

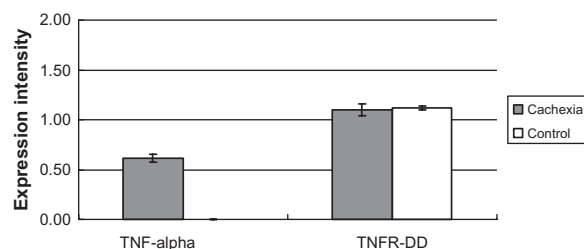


**Figure 4** Intestinal TNF-alpha, sTNFR1, and sTNFR2.

**Notes:**  $P < 0.05$  for TNF-alpha;  $P < 0.01$  for sTNFR1;  $P < 0.001$  for sTNFR2.

**Abbreviations:** TNF, tumor necrosis factor; sTNFR, soluble tumor necrosis factor receptor.





**Figure 5** TNF-alpha and TNFR-DD gene expressions of intestinal mucosa in cachexia mice and control mice.

**Note:**  $P < 0.001$  for TNF-alpha expression intensity relative to GAPDH.

**Abbreviations:** DD, death domain; GAPDH, glyceraldehyde phosphate dehydrogenase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

## Acknowledgments

This study was supported by Harbin Municipal Science and Technology Innovative Fellowship Scheme (#2008RFQXS097).

## Disclosure

The authors report no conflicts of interest in this work.

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