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ORIGINAL ARTICLE

Total parenteral-nutrition-mediated dendritic-cell activation and infiltration into the small intestine in a rat model

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Abstract
Purpose Total parenteral nutrition (TPN) is commonly carried out in the clinical setting. However, effects of TPN on the immune system, including dendritic cells (DC), are not well understood. The purpose of this study was to determine whether TPN affects DC activation and infiltration into the intestinal barrier.
Methods Male Wistar rats were given conventional nutrition (CN) or TPN for 7 days. DCs were visualized by immunohistochemistry. Levels of nucleotide-binding oligomerization domain protein 2 (NOD2) and high-mobility group box 1 (HMGB1) protein were assessed by Western blot.
Results The number of DCs at the small intestinal barrier was significantly increased in the TPN group (9.2 ± 3.1 cells/microscopic field) compared with the CN group (0.5 ± 0.6 cells/microscopic field; $p < 0.05$), as were protein expression levels of NOD2 and HMGB1.
Conclusion These results suggest that TPN increases activation and infiltration of DCs into the small intestine, potentially involving an increase in NOD2 and HMGB1 levels in the small intestine.

Keywords HMGB1 · NOD2 · Nutrition · Intestinal barrier · Immunity

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Introduction

Total parenteral nutrition (TPN) has become a gold standard in supportive care for various types of critically ill patients unable to eat or absorb enterally provided nutrients [1]. However, long-term use of TPN has been shown to be associated with increased incidence of infection [2]. Some experimental studies have demonstrated that intravenous TPN negatively influences gut-barrier functions and mucosal immunity due to withholding nutrients by enteral feeding [3–5].

Dendritic cells (DCs) are antigen-presenting cells that act as sentinels of immunity [6]. DCs are activated by a variety of incoming signals in diverse situations, such as Integra transplantation, cardiopulmonary bypass surgery, strangulated hernias, neonatal necrotizing enterocolitis, and injury to intestinal tissue. In the small intestine, both oral tolerance and priming of immune cells are influenced by the number and activation status of DCs and the lymphoid tissues into which they drain [7]. Our study indicates that dietary proteins are taken up preferentially by DCs in the lamina propria of the small intestine. When intestinal tissue is subjected to hypoxic conditions, DCs orchestrate an immune response. In addition, DCs express receptors for cytokines, chemokines, endogenous danger signals, and microbial structures [6]. Bidirectional interactions between DCs and antigen-experienced T cells initiate either an immunogenic or a tolerogenic pathway and are of crucial importance in autoimmune diseases, infection, and transplantation medicine [8, 9]. DCs also play an essential role in various inflammatory diseases in the small intestine [10]. A recent study also suggests that intestinal DCs are critical for regulation of immunity in the gut [11]. Indeed, antigen uptake in acute inflammation may activate DCs, which then migrate to lymphoid tissue, where they mature. DCs

eventually return to the intestine to participate in the local inflammatory response [12]. DCs, through their ability to orchestrate protective immunity and immune tolerance, play a key role in shaping the intestinal immune response.

High-mobility group box 1 (HMGB1) is a nuclear protein originally identified as an important regulator of genetic information [13]. In 1999, Wang et al. [14] reported that HMGB1 plays a key role as a late-phase mediator in sepsis pathogenesis. HMGB1 is released from activated macrophages and immunostimulated epithelial cells of the gut in a delayed manner relative to secretion of the early proinflammatory mediators, tumor necrosis factor (TNF) and interleukin-1 (IL-1) [15, 16]. HMGB1 is capable of altering gut-barrier function [17]. Moreover, an increase in permeability and bacterial translocation was found to reduce the treatment of anti-HMGB1 antibody from the endotoxemic rat intestine [18]. Thus, HMGB1 may mediate gut-barrier dysfunction and play an important role in intestinal immunity.

Toll-like receptors (TLRs) are key components of the innate immune system. These receptors activate multiple inflammatory pathways that facilitate eradication of invading pathogens [19]. The involvement of toll-like receptor 4 (TLR4) in recognizing the presence of lipopolysaccharide, a major cell-wall component of Gram-negative bacteria, is well established [20]. By responding to bacterial endotoxin and multiple other endogenous ligands, including HMGB1, TLR4 acts at the interface of microbial and sterile inflammation [21]. In vivo evidence for TLR4-mediated danger signaling comes from various studies of acute tissue injury in hemorrhagic shock [22], as well as in cardiac [23], renal [24], and hepatic [25] models of ischemic reperfusion injury. Furthermore, DCs can be activated via TLR4 signaling [26].

Nucleotide-binding oligomerization domain protein 2 (NOD2), which helps trigger host immune defense in response to bacterial peptidoglycans, is a cytosolic protein that plays an essential role in innate immunity. NOD2 fights off bacterial infections by promoting cytokine and chemokine production in immunocytes and enterocytes [27]. A recent study suggested that NOD2 acts cooperatively with molecules sensed by TLR4 to induce the onset of adaptive immune responses, especially DC activation [28, 29]. Moreover, NOD2 binds to the regulatory subunit of the IκB kinase (IKK) complex and leads to nuclear factor kappa B (NF-κB) activation [30]. Thus, NOD2 acts to maintain mucosal homeostasis in tissue damage caused by host modulatory effects in response to commensal bacteria. On the other hand, deficient NOD signaling may cause imbalance in mucosal homeostasis, facilitating injury and leading to disease [31]. NOD2 signaling may stimulate diverse inflammatory responses, leading to acute and chronic intestinal inflammation. The aim of this study was to investigate the mechanism of TPN-induced DC activation and infiltration into the small intestine in a rat model.

Materials and methods

Treatment protocol

All protocols conformed to the National Institutes of Health (NIH) guidelines, and all animals received humane care in compliance with the Principles of Laboratory Animal Care. The study was approved by the Ethical Committee on Animal Research at the College of Medicine, Oita University, Oita, Japan. Male Wistar rats (Kyudou, Saga, Japan) weighing 250–300 g were used in all experiments. Prior to initiation of experiments, all animals were housed with access to food and water ad libitum. Animals were randomly assigned to one of two treatment groups: a conventional nutrition group, or a TPN group. In the conventional nutrition group, the right external jugular vein was catheterized under 4% sevoflurane anesthesia and continuously infused with 2 ml/h saline. Throughout the duration of the experiments, these rats were housed with access to food and water ad libitum. In the TPN group, rats were catheterized as in the conventional nutrition group. Nutritional support was prepared and infused under sterile conditions. The parenteral formulation consisted of TPN solution (Hicaliq NC-H, Terumo, Tokyo, Japan) supplemented with essential and nonessential amino acids (Amiparen 10%, Otsuka, Tokyo, Japan). In addition to various minerals, 1 l of TPN formulation contained 34.5 g of amino acids and 215.5 g of carbohydrates. Rats in the TPN group received approximately 200 kcal/kg per day infused at 1 kcal/ml $\times$ 2 ml/h. Throughout the duration of the experiments, rats of the TPN group were housed with access to water only ad libitum. At 7 days after treatment, all animals were subjected to 3% sevoflurane anesthesia and killed by exsanguination. Small intestinal tissue was removed quickly and processed for histological examination, transmission electron microscopy, immunohistochemical analysis, or Western blotting, as described below.

Histological examination

Small intestinal tissue was fixed using 10% formalin and embedded in paraffin. Tissue samples were sectioned (3 μm) prior to staining with hematoxylin and eosin. The presence and extent of damage to intestinal mucosa were evaluated via light microscopy by two different pathologists according to the criteria of Chiu's method [32]. The Chiu grading system consists of the following five subdivisions based on changes in villi and glands of the intestinal mucosa: grade 0, normal mucosa; grade 1, development of

subepithelial Gruenhagen space at the tip of the villus; grade 2, extension of the space with moderate epithelial lifting; grade 3, massive epithelial lifting with a few denuded villi; grade 4, denuded villi with exposed capillaries; grade 5, disintegration of the lamina propria, with ulceration and hemorrhage.

Transmission electron microscopy

Tissue samples from the small intestine were immersed in Karnovsky fixative solution (glutaraldehyde and formaldehyde) for 2 h at 4°C and cut into small pieces using a razor blade. The specimens were postfixed in 2% osmium for 2 h at 4°C, dehydrated through graded ethanol series, and then embedded in Epok 812. Ultrathin sections (90–95 nm) were prepared with a diamond knife on a Reichert-Nissei Ultracut-S ultramicrotome. The ultrathin sections were examined under a JEM-1200 EX II transmission electron microscope (JEOL, Tokyo, Japan).

Immunohistochemical analysis

Tissue samples from the small intestine were immediately fixed in 4% paraformaldehyde at 4°C overnight, embedded in Optimal Cutting Temperature (OCT) Compound (Sakura Finetechnical Co., Tokyo, Japan), and sectioned at 5 μm. Immunohistochemistry was performed after blocking endogenous peroxidase activity with 0.3% hydrogen peroxide (H_2O_2) and sodium azide (1 mg/ml) for 10 min. Nonspecific protein binding was blocked with 10% sheep serum for 10 min. Blocked sections were incubated with either anti-HMGB1 (Shino-Test Corporation, Tokyo, Japan) or anti-OX62 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) polyclonal antibody (1:1,000 dilution) at 4°C overnight. Sections were rinsed in phosphate-buffered saline (PBS) three times for 5 min per rinse and then incubated with peroxidase-conjugated anti-mouse immunoglobulin G (IgG). After three rinses in PBS, sections were stained using an LSAB2 kit (Dako, Carpinteria, CA, USA) as the biotin–avidin peroxidase complex system. After development, sections were counterstained with Mayer's hematoxylin, mounted, and examined by light microscopy.

Western blotting

Protein extracts were prepared from small intestinal tissue at 7 days after the indicated treatments. Small intestinal tissue was homogenized using T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA), and protein concentrations were assessed using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were prepared for each sample and boiled for 5 min, followed by addition of dithiothreitol. Protein samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA. USA). Membranes were blocked with PBS containing 0.1% Tween-20 (PBS-T) and 5% skim milk for 1 h at room temperature. After three washes with PBS-T, membranes were incubated with HMGB1 (Shino-Test Corporation), NOD2 (Santa Cruz Biotechnology), or actin (Abcam Inc., Cambridge, MA, USA) antibodies (1:1,000 in PBS-T) for 1 h at room temperature. Following three washes in PBS-T, peroxidase-labeled secondary antibodies (1:1,000 in PBS-T; Zymed, San Francisco, CA, USA) were added and incubated for 1 h at room temperature. After three additional washes in PBS-T, membranes were developed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) and exposed on Hyperfilm ECL (Amersham). Actin was used as an internal control. Films were scanned, and densitometry was performed using ImageJ software (National Institutes of Health, USA).

Statistical analysis

DC cells were histologically examined and graded using Chiu's score. The 25th and 75th percentiles of the Chiu's scores are represented as the top and bottom boundaries of the boxes in the box and whisker plots, and the 10th and 90th percentiles are represented as T bars. Densitometry is expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Kruskal–Wallis and Mann–Whitney *U* tests. A p value <0.05 was considered statistically significant.

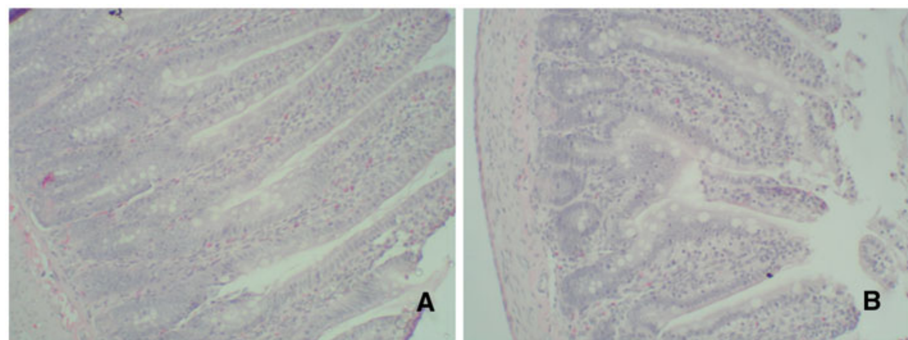
Results

Effects of TPN on histopathology of the small intestine

Small intestinal tissue specimens from the conventional nutrition and TPN groups were collected 7 days after treatment. Whereas normal histology was observed in the conventional nutrition group (Fig. 1a), marked histological changes (multiple erosions, inflammatory cell infiltration, and bleeding) were observed in the TPN group (Fig. 1b). Furthermore, Chiu's score for the conventional nutrition group (mean = 0.4) was significantly lower than in the TPN group (mean = 1.6) ($p < 0.05$; Fig. 1c).

Effect of TPN on DCs in the small intestine

Whereas only a few DCs were detected in the small intestine in the conventional nutrition group (Fig. 2a),



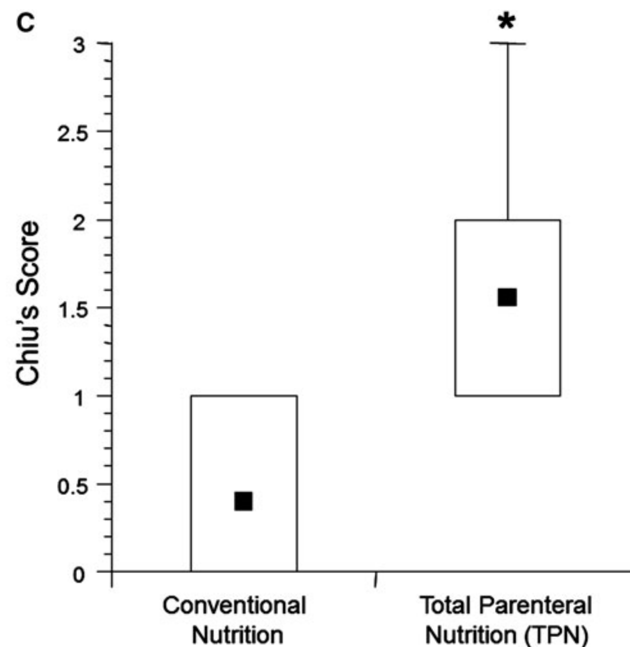


Fig. 1 Effects of total parenteral nutrition (TPN) on histopathology of the small intestine. Small intestinal specimens were obtained 7 days after treatment from the conventional nutrition (**a**) and TPN (**b**) groups and visualized by hematoxylin & eosin (H&E) staining; all photographs are at ×100 magnification. Histological changes are reflected in Chiu's score (**c**). In the *box and whisker plots*, the *square within the box* represents median scores, the *top* and *bottom* boundaries of the boxes represent the 25th and 75th percentiles, respectively, and *T bars* represent the 10th and 90th percentiles. $^{*}p < 0.05$ compared with the conventional nutrition group

significantly more DCs were detected in the TPN group (Fig. 2b).

The following features were used to identify myeloid DCs in the small intestine by electron microscopy: nuclear indentation with peripheral distribution of heterochromatin, abundance of electron-lucent cytoplasm with numerous micropinocytic vesicles, and profiles of endoplasmic reticulum and free ribosomes. Whereas DCs in specimens from the conventional nutrition group had a small and round morphology (Fig. 3a), DCs in the TPN group displayed cell-body extensions and pseudopodia (Fig. 3b).

Effect of TPN on NOD2 and HMGB1 levels in the small intestine

Immunohistochemical analysis revealed that the number of cells expressing HMGB1 in the small intestine increased in the TPN group (Fig. 4b) compared with the conventional nutrition group (Fig. 4a). Furthermore, Western blot analysis revealed dramatically elevated levels of NOD2 and HMGB1 in small intestinal tissue extracts in the TPN group compared with the conventional nutrition group (Fig. 5). Actin levels were the same in both groups (Fig. 5).

Discussion

In this study, we demonstrated that TPN results in damage to the small intestinal epithelium, DC activation, and increased levels of HMGB1 and NOD2 in the small intestine. To the best of our knowledge, this is the first report demonstrating that TPN activates DCs in a rat model.

DCs, as a key link between innate and adaptive immunity, are the most functional antigen-presenting cells that can sense danger signals [6]. DCs function in antigen detection, uptake, and processing, whereas mature DCs primarily function in antigen presentation and cytokine production [33]. Thus, DCs play an important role in maintaining immune homeostasis by regulating initial

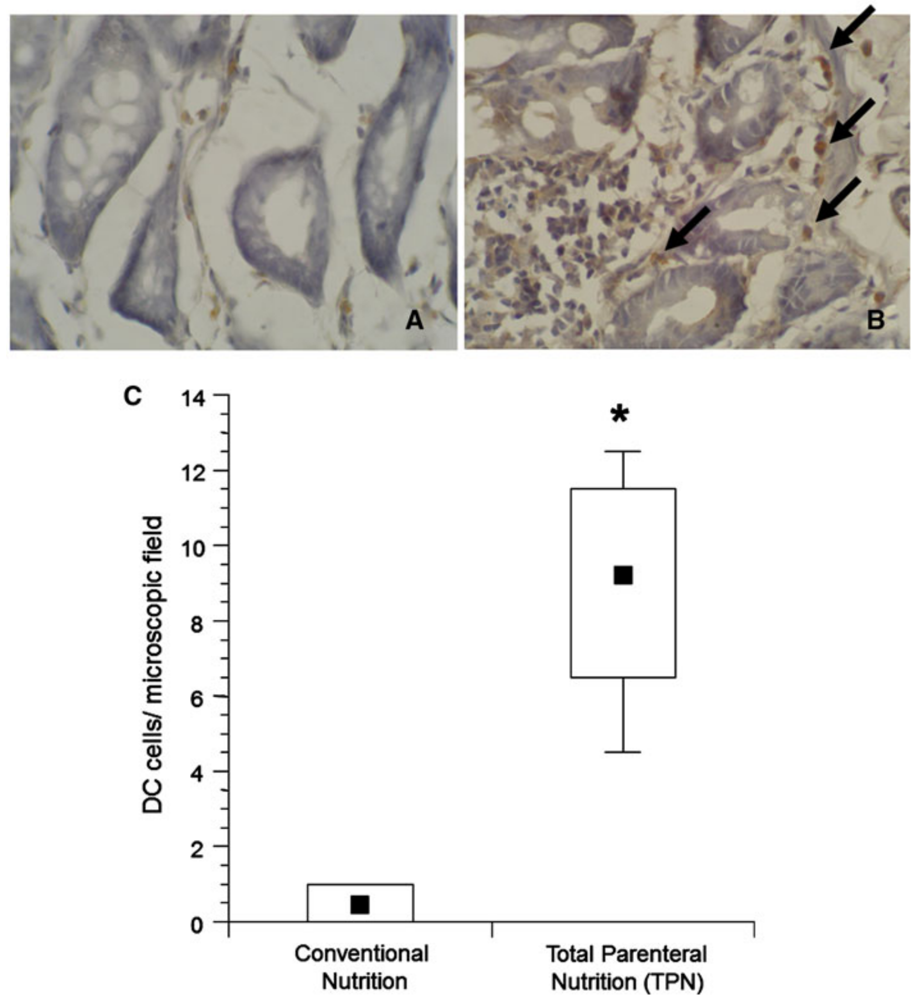


Fig. 2 Increased number of dendritic cells (DCs) in the small intestine. Immunohistochemical analysis was used to detect DCs in small intestinal sections from the conventional nutrition and total parenteral nutrition (TPN) groups 7 days after treatment. DCs were specifically identified by use of anti-OX62 antibodies. All photographs are at ×400 magnification. Representative specimens from the conventional nutrition (**a**) and TPN (**b**) groups are presented. *Arrows* (**b**) show cells that stained positive for OX62, indicating that those cells are DCs. Identified number of DC cells per microscopic field (**c**). In the *box and whisker plots*, the *square within the box* represents median scores, the *top* and *bottom* boundaries represent the 25th and 75th percentiles, respectively, and *T bars* represent the 10th and 90th percentiles. *$p < 0.05$ compared with the conventional nutrition group

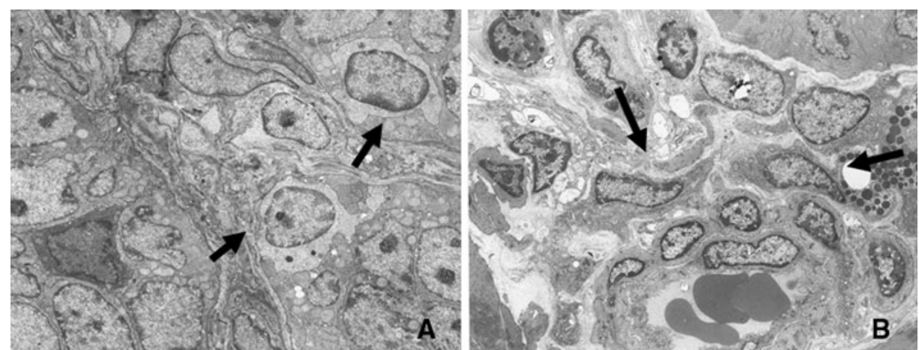


Fig. 3 Effects of total parenteral nutrition (TPN) on dendritic cells (DCs) in the small intestine as assessed by electron microscopy. Tissue specimens were obtained from the conventional nutrition (**a**) and TPN (**b**) groups. All micrographs are at ×6000 magnification. *Arrows* (**a**, **b**) indicate cells positively identified as DCs

responders in a population of damaged or necrotic cells via activation of TLR4 and downstream signaling [34]. DCs not only initiate primary immune responses but also down-regulate immune reactions [35]. Several studies have suggested that DC activation is related to detection and amplification of innate immune responses, thereby promoting the induction of adaptive immune responses [35, 36]. DCs are activated by various signals, and this phenomenon may be related to the production of cytokines and chemokines in response to specific cellular conditions. In particular, necrotic cell death appears to be primarily responsible for recruiting and activating DCs in various tissues [37–39]. Indeed, the lymphocyte-like cells accumulated in the TPN group. Thus, we believe that long-term TPN might evoke inflammation in the small intestine through DC activation. DCs undergo maturation and migrate to draining lymph nodes in response to signals that indicate local “danger” or tissue damage. They also play a

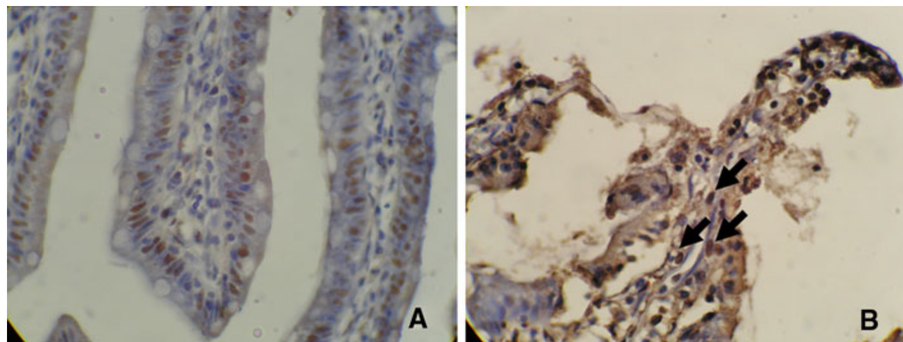


Fig. 4 Total parenteral nutrition (TPN)-mediated increase of high-mobility group box 1 (HMGB1) levels in small intestinal cells. Immunohistochemical analysis was used to examine cells expressing HMGB1 in small intestinal tissue sections of the conventional nutrition and TPN groups. All photographs are at ×400 magnification. Representative specimens from the conventional nutrition (**a**) and TPN (**b**) groups are shown. *Arrows* (**b**) indicate cells staining positive for HMGB1

central role in both stimulating and suppressing immune responses and are impacted by injury, exercise, and other physiological stresses. This study demonstrates that the number of DCs increased in the small intestine in response to TPN. This suggests that DCs may be activated in response to a lack of activity in the small intestine.

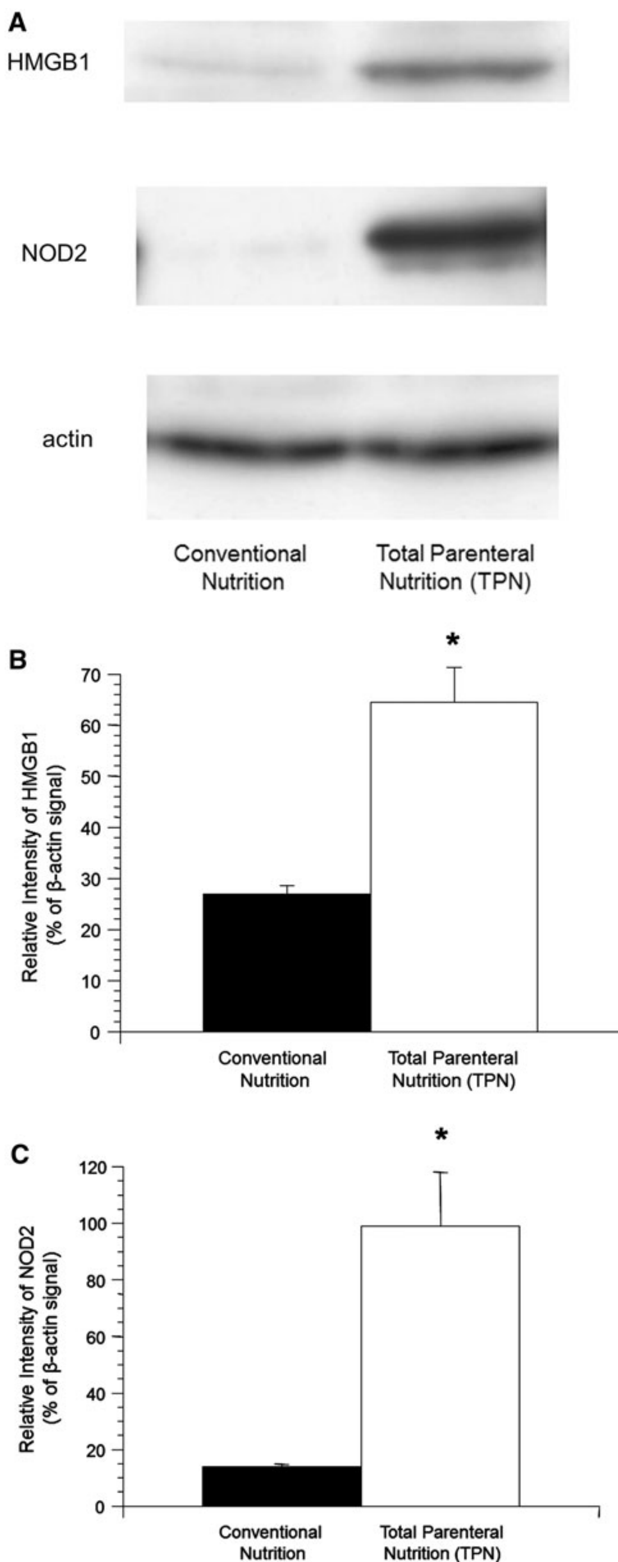
NOD2 is a cytoplasmic protein expressed in various cell types and during the process of intestinal tolerance. NOD2 has also been implicated in intracellular recognition of bacterial components. A lack of NOD2 compromises the function of the gut as a barrier to bacteria [40].

HMGB1 is a nuclear protein originally identified as an important factor in the regulation of genetic information [13]. HMGB1 is a key late-phase mediator in the pathogenesis of sepsis [41], an important mediator in various inflammation models [42], and a key mediator in a variety of other diseases. HMGB1 is released from necrotic cells or secreted by cells such as activated monocytes/macrophages following cytokine stimulation [43]. Secreted HMGB1 then binds to the receptor for advanced glycation end-products (RAGE), Toll-like receptor 2 (TLR2), and TLR4, initiate a signaling cascade that results in further cytokine release and contribute to lethality associated with endotoxemia [14, 44]. Other studies have shown that NOD2 and HMGB1 proteins activate DCs through TLR4 and that DC activation was related to up-regulation of these mediators [28, 45].

Findings from these studies suggest that the use of total parenteral nutrition (TPN) in our study may have induced the expression of HMGB1 and NOD2 proteins, leading to inflammation and damage to the small intestine. We previously reported that TPN enhanced inflammation compared with total enteral nutrition (TEN) [46, 47]. In addition, Ding and Li [48] reported that normal TPN enhanced inflammatory response in the small intestine during systemic inflammation. Thus, we thought this enhanced inflammatory response may have led to an increase in proinflammatory mediators in TPN compared with conventional feeding. Furthermore, DC activation in response to TPN might be related to higher levels of HMGB1 and NOD2 proteins in the small intestine. Thus, therapeutic interventions that inhibit NOD2 or HMGB1 may be effective in minimizing organ damage during conditions of injury to the small intestine in patients. However, we did not pursue the relationship between DC activation and NOD2 and HMGB1 in this study. Thus, further studies will be needed to clarify this issue.

Several limitations to our study should be noted. First, we did not test long-term administration of TPN. Second, we tested the effects of TPN in a rodent model. It is possible that significant differences exist between DCs of the gastrointestinal tract of rodents and humans. It will therefore be necessary to test whether a similar phenomenon exists in humans. Third, we focused our analysis on NOD2 and HMGB1 proteins. One possibility is that after TPN, release of NOD2 and HMGB1 proteins from nucleosomes may contribute to DC activation. Further studies will be required to address this possibility. Given that other important mediators are likely involved in the response to TPN, such as TLRs, further studies will be needed to determine whether they are also involved in this process.

In this study, long-term use of TPN in rats may have induced expression of HMGB1 and NOD2 proteins, leading to inflammation and damage to the small intestine. In addition, the observed DC activation may be related to increased levels of HMGB1 and NOD2 proteins in the small intestine. After long-term use of TPN, the release of NOD2 and HMGB1 proteins may enhance DC TLR4 reactivity, thereby contributing to DC activation. Thus, minimizing TPN may inhibit NOD2 or HMGB1 expression and may be effective in minimizing organ damage in conditions injurious to the small intestine in patients.



◀ **Fig. 5** Changes in high-mobility group box 1 (HMGB1) and nucleotide-binding oligomerization domain protein 2 (NOD2) levels in the small intestine following total parenteral nutrition (TPN). Western blot analysis (**a**) of HMGB1 and NOD2 levels in tissue extracts from the small intestine of conventional nutrition and TPN groups was performed. β-actin serves as an internal control. Densitometry was performed to assess HMGB1 signal intensity in the conventional nutrition and TPN groups (**b**). HMGB1 band intensity is expressed as the percentage of β-actin signal. Data are expressed as mean ±standard deviation (SD). *$p < 0.05$ compared with the conventional nutrition group. Densitometry was performed to assess NOD2 signal intensity in the conventional nutrition and TPN groups (**c**). NOD2 band intensity is expressed as the percentage of β-actin signal. Data are expressed as mean ± SD. *$p < 0.05$ compared with the conventional nutrition group

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Conflict of interest None to report.

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