Homeostasis alteration within small intestinal mucosa after acute enteral refeeding in total parenteral nutrition mouse model

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Feng Y, Barrett M, Hou Y, Yoon HK, Ochi T, Teitelbaum DH. Homeostasis alteration within small intestinal mucosa after acute enteral refeeding in total parenteral nutrition mouse model. Am J Physiol Gastrointest Liver Physiol 310: G273–G284, 2016. First published December 3, 2015; doi:10.1152/ajpgi.00335.2015.—Feeding strategies to care for patients who transition from enteral nutrient deprivation while on total parenteral nutrition (TPN) to enteral feedings generally proceed to full enteral nutrition once the gastrointestinal tract recovers; however, an increasing body of literature suggests that a subgroup of patients may actually develop an increased incidence of adverse events, including death. To examine this further, we studied the effects of acute refeeding in a mouse model of TPN. Interestingly, refeeding led to some beneficial effects, including prevention in the decline in intestinal epithelial cell (IEC) proliferation. However, refeeding led to a significant increase in mucosal expression of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), as well as an upregulation in Toll-like receptor 4 (TLR-4). Refeeding also failed to prevent TPN-associated increases in IEC apoptosis, loss of epithelial barrier function, and failure of the leucine-rich repeat-containing G protein-coupled receptor 5-positive stem cell expression. Transitioning from TPN to enteral feedings led to a partial restoration of the small bowel microbial population. In conclusion, while acute refeeding led to some restoration of normal gastrointestinal physiology, enteral refeeding led to a significant increase in mucosal inflammatory markers and may suggest alternative strategies to enteral refeeding should be considered.

Total parenteral nutrition (TPN), while critically important for the sustenance and survival of patients, has a number of associated adverse problems. Using a mouse model of TPN our laboratory and others have shown that this includes a decline in intestinal epithelial cell (IEC) proliferation and increased IEC apoptosis (18), as well as a marked rise in several mucosal-derived proinflammatory cytokines, like tumor necrosis factor-α (TNF-α), interferon-γ, and interleukin (IL)-6 as well as a downregulation of regulatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) (15, 19, 20). The resultant effect of these changes is intestinal mucosal atrophy and loss of epithelial barrier function (EBF) (27, 32, 36). These changes may have clinical implications, since TPN in humans is associated with increased septic and infectious complications (27). While intuitively refeeding patients after a period of prolonged enteral nutrient deprivation (END) would seem beneficial, the clinical evidence suggests a more complex scenario. Interestingly, a number of recent well-controlled nutritional papers have demonstrated adverse effects of aggressive enteral feeding, particularly in critically ill patients, and this included a significant increase in mortality (25, 26, 42). In fact, nutritional feeding of critically ill surgical patients is complex and often rapidly evolves from states of complete END to the acute introduction of feeds. The implications of the introduction of enteral feedings have not been fully explored. In this study, we hypothesized that the acute provision of enteral nutrition after TPN treatment would reverse TPN-associated loss of IEC proliferation and other TPN-associated changes.

Materials and Methods

Animals. C57BL/6 male, specific-pathogen-free 10-wk-old mice (Jackson Laboratory, Bar Harbor, ME) were maintained under controlled temperature, humidity, and light conditions. Mice were housed in metabolic cages to prevent coprophagia. Studies conformed to our University Committee on Use and Care of Animals (Approval: PR000006293). Catheterized mice initially received 5% dextrose in 0.45 normal saline with 20 meq KCl/l at 4.8 ml/day. After 24 h, mice were randomized to enterally fed control or TPN groups.

TPN model. Protocols are as described previously (46, 49). Enteral controls received intravenous crystallloid standard laboratory chow and water ad libitum. TPN mice received intravenous TPN at 4.8 ml/day. Nitrogen and energy delivery was matched between groups (isonitrogenous/isocaloric) (50). For the refeeding group, full enteral nutrition was reintroduced after 6 days of TPN, and TPN treatment was continued until day 7. All mice were killed 7 days postcannulation with CO2. Mice were killed if their health deteriorated to a level felt unacceptable for the welfare and well-being of the animal, and those mice were not included in the study group. All procedures were in accordance with the University Laboratory Animal Committee.

mRNA expression. Proinflammation cytokine and chemokine mRNA expression was measured with quantitative PCR (qPCR), and gene expression was represented as relative to β-actin, as previously described (12). RNA extraction of mucosal scrapings was as previously described (12, 44). Oligomers were designed using an optimization program (www.premierbiosoft.com).

Western immunoblotting. For protein analysis, a more precise isolation of IEC or IEC nuclear was performed (21). Immunoblots were performed as described (12). Results are expressed as a ratio to β-actin. All primary antibodies were from Cell Signaling Technology, including mouse anti-phospho (p)-Akt, mouse anti-Akt, mouse and rabbit anti-β-catenin, and phospho-β-catenin (Ser552). Secondary antibody was either the corresponding goat anti-mouse horseradish peroxidase or goat anti-rabbit antibody (Santa Cruz).

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We next examined the effect of acute refeeding on the crypt stem cell population using an additional group of Lgr5-EGFR + mice (Fig. 1C). Interestingly, the administration of TPN led to a significant loss of Lgr5-positive cells. However, refeeding, despite the significant restoration in IEC proliferation, did not result in any notable change in this loss of Lgr5 cells (Fig. 1D). This suggested that the rapid restoration of IEC proliferation was occurring in an alternative or potentially a more mature population of stem cells beyond that represented by the Lgr5 + population. More studies may be done in future work.

Proliferative signaling pathways are partially restored with refeeding. We previously described a potential pathway by which the loss of enteral nutrition leads to a significant loss of PI3K/p-Akt signaling and contributes to TPN-associated mucosal atrophy (17). To examine the effect of refeeding, we next measured p-Akt and nuclear β-catenin expression in IEC using Western immunoblotting, also with Wnt and its downstream signaling factors, c-Myc and cyclin D1 mRNA expression. Acute refeeding totally prevented this decline in p-Akt and nuclear β-catenin expression (Fig. 2, A and B). We then examined Wnt and its downstream signaling expression; significant TPN-related decline in Wnt5a, c-Myc, and cyclin D1 expression was confirmed (Fig. 2B), whereas Wnt3 mRNA expression was not affected with TPN treatment. After acute refeeding, cyclin D1 and c-Myc expression were restored to control levels (Fig. 2B), with a dramatic increase in Wnt3 expression (3-fold over control levels). However, Wnt5a failed to change with refeeding. Similar to previous data, TPN administration downregulated TGF-α, epidermal growth factor (EGF), and epidermal growth factor receptor (EGFR) mRNA expression, and the expression of these factors was restored with acute refeeding (Fig. 2C).

Apoptosis persists with refeeding after TPN. EC apoptosis was measured with TUNEL staining (Fig. 3A). Results showed that TPN led to a 10-fold increase in IEC apoptosis vs. enterally fed controls. Surprisingly, unlike proliferation, this high apoptosis index did not reverse in the refeeding group. The apoptosis index persisted at this high level in the acute refeeding group, similar to the TPN group (Fig. 3B).

Mucosal cytokine expression increases its proinflammatory response with refeeding. TNF-α and several other proinflammatory cytokines were measured with real-time PCR, including IL-6, which was found to be upregulated in our TPN mice model. Interestingly, TNF-α expression in refeeding mice rose to even higher levels (4-fold) compared with controls, and IL-6 expression remained similar to the TPN group (Fig. 4A). This trend was also seen with the chemokine monocyte chemoattractant protein 1, which has been reported to play a role in the recruitment of monocytes to sites of injury and infection (39). IL-2 and IL-12b expression were not affected with TPN treatment; however, IL-2 and IL-12b expressions were both upregulated after acute refeeding in TPN mice (Fig. 4A).

T regulator cell-produced cytokines IL-10 and TGF-β are known to modulate the proinflammatory response with TPN (20) and can sustain IEC proliferation and apoptosis (41). In our TPN model, it had been reported that TPN administration decreased IL-10 and TGF-β expression. With acute refeeding, the decline in TGF-β was totally reversed, and IL-10 was partially reversed (Fig. 4B).

Mucosal microbiota alteration with refeeding. Both Toll-like receptor (TLR) 4 and 5 expression was increased with
TPN treatment; interestingly, both were further increased in the refeeding group compared with the TPN group (Fig. 4C). Potentially, this may be due to a major shift in the microbial population of the small bowel from a Firmicutes-dominant toward a gram-negative Proteobacteria-dominant population with TPN administration (31). Interestingly, TLR-4 can signal in response to gram-negative bacteria lipopolysaccharides, and the expression of TLP4 rose fourfold higher after refeeding compared with the TPN group (31).
We then examined the microbiome population in our refeeding group using 454 pyrosequencing. The results showed a partial return of the Firmicutes population, but a persistent increase in Proteobacteria and Bacteroidetes, as well as a variety of other bacterial species (Fig. 5A).

Acute refeeding after TPN did not restore paneth cell function. Paneth cells are located in the base of small intestinal crypts. These cells produce α-defensin, lysozyme, and matrix metalloproteinase 7 (MMP7), which have anti-bacterial functions and limit the number of bacteria that localize at the...
mucosal surface, in or beneath the mucus layer. It has been previously reported that TPN leads to a loss of Paneth cell function (23). In the present study, H9251-defensin (cryptins) expression was measured with qPCR. Cryptins 2–6 were significantly downregulated in the TPN mice. No change in the abundance of cryptins was found in the refeeding group (Fig. 5B).

MMP7 converts inactive pro-α-defensins to bactericidal forms by proteolysis at specific pro-region cleavage sites. We next examined for MMP7 expression. The results showed that TPN led to a marked decline in Paneth cell MMP7 protein expression compared with the controls. Refeeding failed to reverse this change (Fig. 5C). The Paneth cell marker lysozyme was detected at the immunofluorescence level. The abundance of its expression was markedly downregulated in the TPN group, and refeeding partially reversed this decline (Fig. 5D).

Epithelial barrier function failed to improve with acute refeeding. A major consequence of TPN administration is a loss of EBF (45). We next measured TER in TPN mice. This led to a decreased TER, similar to what we have previously reported, to about 14.77 ± 1.67 Ω·cm² compared with controls (30.13 ± 2.63 Ω·cm²). Acute refeeding just increased TER a little bit to 18.83 ± 2.23 Ω·cm² (Fig. 6A) (15). FITC-dextran 40 (FD40) permeation was tested with effluent. There was very low FD40 tested in the control group (4.10 ± 2.75 μg/ml). However, TPN increased the concentration to 14.49 ± 4.85 μg/ml. Importantly, acute refeeding did not protect this, but, rather, the FD40 concentration remained elevated at 11.64 ± 4.51 μg/ml (Fig. 6B).

The tight junction molecule zonula occludens (ZO)-1 and occludin mRNA abundance was measured (Fig. 6C). TPN led to decreased mRNA expression of both molecules, and expression of these was totally reversed in the refeeding group, leading to ZO-1 and occludin gene expression to even higher compared with the control group. However, immunofluorescence staining showed that TPN administration led to decreased ZO-1 and occludin expression at the protein level. Furthermore, while the density of ZO-1 and occludin staining was partially increased in the refeeding group compared with

Fig. 3. IEC apoptosis persists with refeeding after TPN. A: TdT-dUTP nick end-labeling (TUNEL) staining was performed to detect IEC apoptosis. B: apoptosis index was calculated as the ratio of TUNEL-positive cell number to total cell number in each villi; 15 villi were selected for each section. Results are means ± SD. For each experiment, n = 5–6 for each group. ***P < 0.001.
Fig. 4. Mucosal cytokine and Toll-like receptor (TLR) expression increases its proinflammatory response with acute refeeding. A: mucosal proinflammatory cytokine expression was measured with real-time PCR. B: regulatory cytokines IL-10 and TGF-β expression were measured with real-time PCR. C: mucosal Toll-like receptor expression. MCP1, monocyte chemoattractant protein 1. Results are means ± SD. For each experiment, n = 5–6 for each group. *P < 0.05, **P < 0.01, and ***P < 0.001.
Fig. 5. Mucosal microbiota and defensin expression with acute refeeding. A: phylum level analysis after RDP classification of pyrosequenced ileal mucosa-associated bacteria samples. B: α-defensin (cryptins) expression was measured with quantitative PCR (qPCR), corrected for β-actin expression. C: matrix metalloproteinase 7 (MMP7) expression (red) with DAPI (blue nuclear counterstain) was measured with immunofluorescence staining. D: lysosome expression (red) with DAPI (blue nuclear counterstain) and PCNA (green) was measured with immunofluorescence staining. Results are means ± SD. For each experiment, n = 5–6 for each group. **P < 0.01.

**REFEEDING AFTER TPN AUGMENTS MUCOSAL INFLAMMATION**

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Fig. 6. Epithelial barrier function failed to improve with acute refeeding. A: transepithelial resistance (TER) was measured using Ussing chambers. B: permeability was measured with FITC-dextran-40 (FD40) concentration. C: zonula occludens (ZO)-1 and occludin expression was measured with real-time PCR and immunofluorescence staining. D: claudin-2 expression was measured with real-time PCR and immunofluorescence staining. Results are means ± SD. For each experiment, n = 5–6 for each group. *P < 0.05, **P < 0.01, and ***P < 0.001.
TPN mice, acute refeeding actually led to a marked increase in the amount of cytoplasmic internalization of ZO-1 and occludin. In summary, these architectural changes demonstrate that refeeding led to a further deterioration of tight junction integrity.

The pore-forming protein claudin-2 (Fig. 6C), which has been associated with other proinflammatory conditions that have a loss of EBF, such as Crohn’s disease (28), was increased in the TPN group compared with the control group at the mRNA level. Interestingly, at both the mRNA level and by immunofluorescence staining, claudin-2 markedly increased in the refeeding group (Fig. 6C and Table 1).

IEC proliferation, morphology, and expression of IEC stem cells returned to enterally fed levels 72 h post-TPN refeeding. While a complete evaluation of other time periods post-TPN was felt beyond the scope of the current work, we next measured several IEC proliferative, apoptosis measures, and TER, as well as the presence of Lgr5+ stem cells 72 h postenteral refeeding (Table 2). Interestingly, all of these measures reversed; the mice did not lose more body weight than the mice on TPN, but they also did not gain body weight with acute refeeding. Animals refed for 72 h start to gain body weight (data not shown), suggesting that the sudden introduction of feeding does lead to an adverse proinflammatory state, but the effects are transient, and will reverse with time. How-

### Table 1. Intestinal epithelial cell proliferation and apoptosis rates and mRNA expression of key regulatory factors and junctional markers

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>TPN</th>
<th>TPN + Refeeding</th>
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<tr>
<td><strong>Intestinal epithelial cell cycle</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PCNA</td>
<td>0.51 ± 0.06***</td>
<td>0.17 ± 0.05</td>
<td>0.42 ± 0.06***</td>
</tr>
<tr>
<td>BrdU</td>
<td>0.45 ± 0.03***</td>
<td>0.19 ± 0.06</td>
<td>0.37 ± 0.08***</td>
</tr>
<tr>
<td>Apoptosis index (TUNEL)</td>
<td>0.012 ± 0.004***</td>
<td>0.102 ± 0.009</td>
<td>0.11 ± 0.006</td>
</tr>
<tr>
<td><strong>Proliferation regulation (mRNA)</strong></td>
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<tr>
<td>Lgr5</td>
<td>0.0088 ± 0.0047**</td>
<td>0.0016 ± 0.0034</td>
<td>0.0031 ± 0.0035</td>
</tr>
<tr>
<td>EGF-REceptor</td>
<td>0.0006 ± 0.0018**</td>
<td>0.0035 ± 0.0078</td>
<td>0.0094 ± 0.0133**</td>
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| Epiregulin                                    | 0.00053 ± 0.00021*| 0.00020 ± 0.00025| 0.00207 ± 0.0083***
| TGF-α                                        | 0.014 ± 0.005*  | 0.0069 ± 0.002  | 0.021 ± 0.005***|
| Wnt3                                         | 0.001 ± 0.004*  | 0.0006 ± 0.001  | 0.0032 ± 0.0011**|
| Wnt5a                                        | 0.0039 ± 0.0011**| 0.0020 ± 0.0005| 0.0027 ± 0.00095|
| c-MYC                                        | 0.025 ± 0.012*  | 0.0097 ± 0.0045| 0.014 ± 0.0068  |
| Cyclin D1                                     | 0.016 ± 0.0084* | 0.0064 ± 0.0009| 0.016 ± 0.0041*|
| **Cytokine/chemokines (mRNA)**               |                 |                |                 |
| TNF-α                                        | 0.0003 ± 0.0008**| 0.00043 ± 0.0004| 0.00059 ± 0.0004***|
| IL-6                                         | 0.00025 ± 0.0013***| 0.00055 ± 0.00007| 0.00066 ± 0.00007|
| MCP1                                         | 0.0011 ± 0.00061 | 0.0017 ± 0.00046| 0.0031 ± 0.0018*|
| TLR4                                         | 0.00048 ± 0.00006*| 0.00070 ± 0.00006| 0.0019 ± 0.0009**|
| TLR5                                         | 0.00045 ± 0.00018*| 0.00086 ± 0.00015| 0.0016 ± 0.0008* |
| IL-10                                        | 0.00062 ± 0.0003*| 0.00023 ± 0.00006| 0.00048 ± 0.00009|
| TGF-β                                        | 0.0033 ± 0.002*  | 0.0018 ± 0.0008| 0.0048 ± 0.0018*|
| **Tight junction markers (mRNA)**            |                 |                |                 |
| E-cadherin                                    | 0.29 ± 0.08*    | 0.16 ± 0.05    | 0.23 ± 0.11     |
| ZO-1                                          | 0.041 ± 0.008*  | 0.024 ± 0.003  | 0.068 ± 0.0177**|
| Occludin                                      | 0.067 ± 0.018** | 0.022 ± 0.011  | 0.114 ± 0.034** |
| Claudin 1                                     | 0.00048 ± 0.00019| 0.00027 ± 0.00011| 0.00037 ± 0.00005|
| Claudin 2                                     | 0.0253 ± 0.00083*| 0.0065 ± 0.0014| 0.0146 ± 0.0052***
| JAM1                                         | 0.103 ± 0.039   | 0.084 ± 0.01   | 0.141 ± 0.015** |
| TJP1                                          | 29.52 ± 2.63*** | 15.2 ± 1.29    | 19.42 ± 2.09**  |

Values are means ± SD; n = minimum of 5 mice/group. TPN, total parenteral nutrition; PCNA, proliferating cell nuclear antigen; BrdU, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-DUTP nick end-labeling; Lgr5, leucine-rich repeat-containing G protein-coupled receptor 5; EGF, epidermal growth factor; TGF, transforming growth factor; IL, interleukin; TLR, Toll-like receptor; ZO-1, zona occludens-1; lAM1, junctional adhesion molecule 1; TER, transepithelial resistance. See MATERIALS AND METHODS for description of expression of intestinal epithelial cell (IEC) proliferation and apoptosis measures. Expression of RNA abundances was adjusted for expression of β-actin. Results are compared with the TPN group: *P < 0.05, **P < 0.01, and ***P < 0.001 via ANOVA.

### Table 2. Intestinal physiological and morphological measures after refeeding for 72 h post-TPN

<table>
<thead>
<tr>
<th>Measure</th>
<th>Sham</th>
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</tr>
<tr>
<td>BrdU</td>
<td>0.32 ± 0.11</td>
<td>0.34 ± 0.07</td>
</tr>
<tr>
<td>TUNEL</td>
<td>0.011 ± 0.004</td>
<td>0.016 ± 0.004</td>
</tr>
<tr>
<td>Villus length, μm</td>
<td>452.9 ± 73.3</td>
<td>392.8 ± 84.3</td>
</tr>
<tr>
<td>Crypt depth, μm</td>
<td>83.4 ± 14.1</td>
<td>78.5 ± 15.1</td>
</tr>
<tr>
<td>Lgr5-EGFP+ IEC number/100 crypts</td>
<td>21.16 ± 1.13</td>
<td>18.70 ± 1.21</td>
</tr>
<tr>
<td>TER, Ω cm⁻²</td>
<td>30.6 ± 2.8</td>
<td>29.1 ± 4.3</td>
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</table>

Results are means ± SD; n = minimum of 4 mice/group. Physiological and morphological measures after refeeding for 72 h post-TPN. Note no significant differences were detected between the sham group and the 72 h refeeding group. Immunofluorescence staining for both PCNA and BrdU was used to measure IEC proliferation, and TUNEL staining was used for detection of IEC apoptosis (see MATERIALS AND METHODS).

**DISCUSSION**

When clinically feasible, enteral feeding provides the ideal method of nutrient delivery (2, 29, 33, 34, 48). Even among...
critically ill patients, where “bowel rest,” or the withholding of enteral feeding, has been historically practiced, the early initiation of feeds within 48 h of intensive care unit (ICU) admission has been shown to reduce mortality and costs compared with delayed feeding (7, 8). When enteral feeding is not possible, however, the parenteral route becomes a life-sustaining method of delivering calories and nutrients. TPN, wherein enteral nutrient delivery is fully withheld and all nutrition is supplied intravenously, is required for over 350,000 patients per year in the United States (35). Of these, over 10% require home-based, long-term TPN. Clearly, TPN has become a cornerstone of modern surgical and critical care. Although it is life preserving, however, TPN is not without substantial risk.

This study identified several important findings occurring within the small bowel mucosa with the reintroduction of enteral feeding. This included a reversal in the decline in IEC proliferation, which may be possibly driven via p-AKT-EGF signaling and stimulation of the Wnt pathway to stimulate the stem cell turnover in the small intestine. A failure of expansion in the Lgr5+ stem cell population with acute refeeding is quite interesting. It may well be that a slower cycling stem cell, such as Bmi1 and mTert populations, was more responsive to acute enteral nutrition. However, acute refeeding led to a marked increase in several mucosal inflammatory factors. This proinflammatory state may well act to counter some of the beneficial effects of enteral feeding after long-term TPN. It was noted that acute introduction of enteral nutrition failed to prevent the observed increase in IEC apoptosis. It is likely that the increased proinflammatory expression of TNF-α may have prevented the TPN-associated increase in IEC apoptosis, since this is very tightly linked to the TPN-associated IEC apoptosis (14). We have previously shown a critical link to this proinflammatory response and a loss of EBF (15, 16). Thus, it is quite possible that the persistent loss of EBF in the refeeding group was due to the increase in the expression of this proinflammatory response. Individual TLRs recognize conserved microbial products. In our study, the alteration of mucosal microbiota changed TLR expression and continued to upregulate TLR expression after acute refeeding. TPN decreased Paneth cell function in the Lgr5+ stem cell population in the TPN and refeeding model. These data may help to provide a new strategy for refeeding patients. More mechanisms may be explored in future study. Our model does not totally mimic the clinical procedure in some institutions. The practice of feeding after a period of TPN varies greatly and is instituted in various ways. Our data indicate that the simple process of refeeding a patient may be far more complex than previously perceived.

In the critical care setting, the use of early parenteral nutrition has been well-demonstrated to benefit patients who would otherwise have not received enteral nutrition until day 3 of ICU admission, resulting in reduced mechanical ventilation needs, improved quality of life, and reduced costs (9, 10). These findings have recently been confirmed in the CALORIES trial, a large randomized trial that found no significant difference in infectious complications or 30-day mortality between early initiation of parenteral vs. enteral nutrition among the critically ill (22). These findings may be due to the low rate of delivering full caloric and nutrient needs via the enteral route alone among ICU patients, who are prone to feeding intolerance and feeding delays from procedures. These findings support the premise that, when adequate nutrition cannot be delivered adequately via the enteral route, parenteral nutrition is a necessary intervention.

Outside of the critical care setting, the risks of TPN become increasingly significant, with metabolic derangements, hepatic dysfunction, and systemic infection posing the most significant risks (2, 4, 24, 33, 34, 42a). A seminal Veterans Affairs study found that patients with mild or moderate malnutrition had an increased rate of infectious complications, including urinary tract infections, wound infections, and pneumonia (42a). Importantly, these complications were not due simply to central line infections, but instead implicated a systemic propensity for developing multisite infections. As the duration of TPN dependence increases, so does the associated risk of infectious complications. For example, children requiring long-term TPN due to intestinal failure have up to a 27% cumulative mortality, with hepatic failure and systemic infections contributing significantly to this rate (5, 40). The clinical management of TPN-dependent patients, therefore, focuses on reevaluating the need for TPN and advancing enteral feeding as tolerated.

There were limitations to the present study that will need to be approached in future investigation. First, while the analysis was quite detailed, the mechanisms that may be driving these proinflammatory changes have not been determined. One potential is that enteral nutrition may actually lead to a marked expansion of a potentially adverse or virulent group of organisms (e.g., Proteobacteria) that can signal via TLR-4 (which was increased with refeeding). Another limitation was the arbitrary time selected after refeeding. Clearly, while beyond the scope of this work, a time line of changes may prove useful. This future work may also demonstrate an evolving change in the microbiome with the progression of enteral feeding, but may also guide clinicians to develop safer approaches to enteral feedings.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Y.F. and D.H.T. conception and design of research; Y.F., M.B., Y.H., H.K.Y., and T.O. performed experiments; Y.F. analyzed data; Y.F. and D.H.T. interpreted results of experiments; Y.F. prepared figures; Y.F. and D.H.T. drafted manuscript; Y.F. and D.H.T. edited and revised manuscript; Y.F. and D.H.T. approved final version of manuscript.

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