Glutamine Improves Innate Immunity and Prevents Bacterial Enteroinvasion During Parenteral Nutrition

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Abstract

Background: Patients receiving parenteral nutrition (PN) are at increased risk of infectious complications compared with enteral feeding, which is in part explained by impaired mucosal immune function during PN. Adding glutamine (GLN) to PN has improved outcome in some clinical patient groups. Although GLN improves acquired mucosal immunity, its effect on innate mucosal immunity (defensins, mucus, lysozymes) has not been investigated. *Methods*: Forty-eight hours following venous cannulation, male Institute of Cancer Research mice were randomized to chow (n = 10), PN (n = 12), or PN + GLN (n = 13) for 5 days. Small intestine tissue and luminal fluid were collected for mucin 2 (MUC2), lysozyme, cryptdin 4 analysis, and luminal interleukin (IL)–4, IL-10, and IL-13 level measurement. Tissue was also harvested for ex vivo intestinal segment culture to assess tissue susceptibility to enteroinvasive *Escherichia coli. Results*: In both luminal and tissue samples, PN reduced MUC2 and lysozyme ($P < .0001$, respectively) compared with chow, whereas GLN addition increased MUC2 and lysozyme (luminal, $P < .05$; tissue, $P < .0001$, respectively) compared with PN alone. PN significantly suppressed cryptdin 4 expression, while GLN supplementation significantly enhanced expression. IL-4, IL-10, and IL-13 decreased significantly with PN compared with chow, whereas GLN significantly increased these cytokines compared with PN. Functionally, bacterial invasion increased with PN compared with chow ($P < .05$), while GLN significantly decreased enteroinvasion to chow levels ($P < .05$). *Conclusions*: GLN-supplemented PN improves innate immunity and resistance to bacterial mucosal invasion lost with PN alone. This work confirms a clinical rationale for providing glutamine for the protection of the intestinal mucosa. (*JPEN J Parenter Enteral Nutr*. XXXX;xx:xx-xx)

Clinical Relevancy Statement

The addition of GLN to nutrition support is of interest in optimizing immunity and recovery that can become compromised during metabolic stress. In the intestine, the mucosa is protected by aspects of adaptive and innate immune function. While the effect of GLN during PN has been investigated in regard to adaptive immunity, such as sIgA and lymphocyte levels, this is the first work to examine the how GLN might maintain innate barrier defenses of the intestine during PN.

Introduction

Parenteral nutrition (PN) provides specialized nutrition support to patients with contraindications to enteral feeding. However, PN administered to postsurgical/trauma and critically ill patients results in an increased risk of infectious complications compared with patients fed enterally.[1-4] These PN-induced vulnerabilities are undoubtedly multifactorial, but impaired mucosal defenses, including innate and acquired immunity, likely contribute to the observed infectious morbidity.[5] While enteral feeding remains the preferred method of nutrition support, some patients cannot be fed enterally, so the availability of a parenteral nutrient supplement that would stimulate mucosal immune defenses could

be clinically advantageous; one supplement showing clinical promise is glutamine (GLN).

GLN serves as a primary metabolic nutrient for the intestinal mucosa during stress and provides an important energy substrate for rapidly proliferating cells, such as enterocytes and

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immune cells, including T lymphocytes. PN with lack of enteral nutrition (EN) negatively affects adaptive immunity, through a reduction in gut-associated lymphoid tissue (GALT) lymphocytes and immunoglobulin A (sIgA) production. Our previous work demonstrates that GLN supports acquired mucosal defenses during PN. The addition of GLN to PN attenuates impaired acquired immunity following PN alone by preventing reductions in GALT cellularity—including the Peyer patches, lamina propria, and intraepithelial cell populations—maintaining IgA-stimulating cytokines, and preserving intestinal and respiratory IgA levels.[6-8] Moreover, compared with PN alone, the addition of GLN to PN prevented impairment in upper respiratory tract immunity to the H1N1 virus and *Pseudomonas aeruginosa*.[9,10] No work has focused on the effect of GLN upon innate immunity during PN.

Innate immunity plays an important role in protecting the gastrointestinal (GI) tract mucosal surfaces. Specialized secretory epithelial cells, specifically goblet cells and Paneth cells within the mucosa, constitute the cellular components of innate mucosal immunity. A mucus layer consisting of glycoproteins released from the goblet cells coats the mucosal surface, concentrates the sIgA produced by the GALT (acquired immunity), and localizes antimicrobial compounds, such cryptdin 4 and lysozymes produced and released by the Paneth cells. This defensive layer forms a physiochemical layer against foreign antigens and pathogenic microorganisms.[11,12] Cryptdin 4 exhibits bactericidal activity against both gram-positive and gram-negative bacteria and displays the greatest antimicrobial activity compared with other crypdins.[13-15] Lysozyme provides antimicrobial activity against gram-positive bacteria, but its function is potentiated in the presence of other gram-negative bactericidal molecules.[14,16] Deficiency in cryptdin 4, lysozyme, or mucin 2 (MUC2) results in susceptibility to enterocolitis and intestinal infections in mice and humans.[17-20] In our previous work, PN reduced messenger RNA (mRNA) expression of cryptdin 4 and other antimicrobial molecules as well as the secretion of MUC2, which impairs the intestinal immune barrier function and increases susceptibility to bacteria invasision.[21,22] Others have shown that GLN supplementation during PN attenuates impaired intestinal permeability and intestinal inflammation in mice.[23,24]

The benefit of GLN is likely in part through modulation of intestinal cytokine levels. GLN increases Th2 lymphocyte-derived cytokines—interleukin (IL)–4, IL-10, and IL-13—in small intestinal tissue homogenates compared with PN alone.[25,26] IL-4 and IL-10 stimulate IgA secretion by plasma cells in support of adaptive immune function, but these cytokines also support innate immunity. IL-4 induces differentiation of glycoprotein-containing goblet cells, and injection of IL-10 into enteric tissue induces goblet cell hyperplasia.[27] IL-10–deficient mice have lowered MUC2 synthesis and structurally altered goblet cells due to impaired MUC2 protein folding responses, which leads to lethal colitis upon bacterial challenge.[28] Accordingly, IL-10 protects animals from developing lethal intestinal infections following parasitic challenge.[29] Both

IL-4 and IL-13 increase MUC2 production in intestinal goblet cells.[30] In addition, IL-13 induces Paneth cell hyperplasia and upregulates gene expression of cryptdin 4.[31] Our prior work demonstrated that stimulating Th2 cytokines with IL-25 maintained innate immunity by increasing the Paneth cell antimicrobial protein, $sPLA_2$, and MUC2 production.[21]

With this substantial experimental background, we hypothesized that addition of GLN to PN would improve innate immunity by maintaining the Th2 response, preserving the production of antimicrobial peptides cryptdin 4, lysozyme, and glycoprotein MUC2. Moreover, we speculated that the addition of GLN would increase intestinal barrier function against bacterial enteroinvasion using an ex vivo intestinal segment culture (EVISC) compared with PN alone.

Materials and Methods

Animals

All animal experiment protocols were approved by Animal Care and Use Committee of the University of Wisconsin–Madison and the William S. Middleton Memorial Veterans Administration (VA) Hospital, Madison. Male Institute of Cancer Research (ICR) outbred mice were purchased from Harlan (Indianapolis, IN) and housed 5 per covered/filtered box in an American Association for Accreditation of Laboratory Animal Care–accredited conventional facility on the William S. Middleton Memorial VA Hospital Campus. The mice were fed standard mouse chow (Rodent Diet 5001, LabDiet; PMI Nutrition International, St Louis, MO) and water under a controlled temperature, humidity, and light cycle (a 12:12-hour light/dark cycle) for 1 week (acclimatization) prior to initiation of the study protocol.

Experimental Design

In experiment 1, 35 male ICR mice (6–8 weeks old) were randomized to receive standard chow (chow, n = 10), PN (n = 12), or PN supplemented with glutamine (2%, PN + GLN, n = 13). Animals were anesthetized with an intraperitoneal administration of a mixture of ketamine (100 mg/kg body weight) and acepromazine (10 mg/kg body weight). A silicone rubber catheter (0.012-inch inner diameter by 0.025-inch outer diameter; Helix Medical, Inc, Carpinteria, CA) was placed into the vena cava through the right jugular vein for intravenous (IV) infusion, and the distal end of catheter was tunneled subcutaneously over the back to exit the midpoint of the tail. The mice were partially restrained by the tail to protect the catheter during infusions. This partial-restraint technique does not induce significant stress in the mice.[32] Then, 0.9% saline was infused to all mice at a rate of 4 mL/d with free access to water and chow for 2 days after surgery. On day 3, animals were assigned to their respective treatments. Chow mice received 4 mL/d of IV 0.9% saline along with ad libitum chow and water. The PN and PN + GLN mice initially received 4 mL/d of their respective solutions on the third day and advanced to 7 mL/d on the fourth day and

10 mL/d for the remaining 3 days. The formulation of PN solution was described previously containing 6.0% amino acids and 35.6% dextrose, electrolytes, and multivitamins, with 1440 kcal/L and a nonprotein calories/nitrogen ratio of 128:1. The PN + GLN solution replaced 2% of the amino acid solution with L-glutamine (Ajinomoto North America, Raleigh, NC). The PN and PN + GLN solutions were nearly isocaloric and isonitrogenous, and they met the calculated nutrient requirements of mice weighing 25–30 g. This GLN dose was chosen because a 30-g mouse receiving 2% glutamine at rates of 4 mL/d (day 1), 7 mL/d (day 2), and 10 mL/d (days 3–5) will receive the metabolically scaled (7.1:1) levels of 0.38 g/kg/d, 0.66 g/kg/d, and 0.94 g/kg/d, respectively, while a typical 70-kg human may receive 30 g/d GLN in the clinic (0.43 g/kg/d).

After 5 days of feeding (7 days after catheterization), mice were weighed, anesthetized as described above, and exsanguinated via left axillary artery transection. The small intestine was removed and the lumen rinsed with 20 mL buffer (HBSS; Bio Whittaker, Walkersville, MD). The luminal wash was centrifuged at 2000 *g* for 10 minutes, and supernatant was aliquoted and frozen at −80°C for analysis of MUC2, lysozyme, and cytokines levels. Ileal tissue samples were taken from a 3-cm segment of ileum that excluded Peyer patches. These samples were stored at −80°C until subsequent analysis. The tissue samples for goblet cell and Paneth cell immunohistochemistry analysis were fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, and stored at 4°C until subsequent histology.

In experiment 2, a total of 22 male ICR mice (6–8 weeks old) were randomized to receive standard chow (chow, n = 8), PN (n = 7), or PN supplemented with glutamine (2%, PN + GLN, n = 7). All the procedures are the same as experiment 1. Two distal ileum segments of 2 cm excluding Peyer patches for each mouse were collected for the EVISC methodology to determine bacterial enteroinvasion.

Periodic Acid–Schiff Staining

The ileum tissue samples were fixed in formaldehyde, processed (Tissue-Tek V.I.P.; Sakura Finetek, Torrance, CA), and embedded in paraffin, after which tissue sections (5 μm thick) were cut. Periodic acid–Schiff (PAS) staining was performed as previously reported.[33] Goblet cells were counted by determining the average number of goblet cells in 15 individual villi in representative active microscopic fields (original magnification, ×20) per mouse.

Western Blot for MUC2 in the Small Intestinal Wash Fluid and Ileum Tissue

Western blotting was performed as previously described.[33] Briefly, small intestinal wash fluid (SIWF) was separated on a 4%–15% polyacrylamide gel (Ready Gel; Bio-Rad Laboratories, Hercules, CA) by electrophoresis. After transfer, membranes were equilibrated in TBS-Tween (Tris-buffered saline with 0.5% Tween-20) for 10 minutes and blocked (5% nonfat dry milk) for 1 hour at room temperature. The membranes were incubated overnight at 4°C with the primary antibody mouse anti–human MUC2 (ab-11197; Abcam, Cambridge, MA) diluted 1:2500 in blotto. Goat anti–mouse IgG–horseradish peroxidase (HRP) conjugate (sc-2005; Santa Cruz Biotechnology, Santa Cruz, CA) antibody was diluted in blotto (ratio: 1:20,000) and incubated for 1 hour with constant rocking. Membranes were detected using HRP substrate (Super Signal West Femto maximum sensitivity substrate; Pierce, Rockford, IL), and the membrane was scanned using Image Quant LAS 4000 (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). Densitometric analysis of target bands at the monomeric and dimeric MUC2 forms was determined by National Institutes of Health (NIH, Bethesda, MD) Image J software. The internal controls were used to normalize the density among multiple membranes.

The tissue protein was homogenized from 3 cm of a frozen small intestine segment using RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1% protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). The homogenate was incubated for 20 minutes on ice followed by centrifugation at 16,000 *g* at 4°C for 10 minutes. The supernatant was stored at −80°C until analysis. The protein concentration of the supernatant was determined by the Bradford method using bovine serum albumin (BSA) as a standard; 20 μg of total tissue protein was used to quantify ileum tissue MUC2 as described above.

Western Blot for Lysozyme in the Small Intestinal Wash Fluid and Ileum Tissue

In total, 20 μL of SIWF was heated at 95°C for 5 minutes, separated on a 10% polyacrylamide gel (Ready Gel; Bio-Rad Laboratories, Hercules, CA) by electrophoresis at 150 V for 50 minutes, and transferred to a PVDF membrane (0.45 μm) using the standard transfer buffer at 80 V for 30 minutes. After blocking in blotto as described above, the membranes were incubated with the primary antibody rabbit anti–Lysozyme (ab-108508; Abcam) diluted 1:5000 in blotto overnight at 4°C. Goat anti–rabbit IgG-HRP conjugate (32260; Pierce) was diluted (ratio 1:15,000) for 1 hour at room temperature. Membranes were detected with HRP substrate (Super Signal West Femto maximum sensitivity substrate; Pierce) for 5 minutes, and the membranes were scanned using Image Quant LAS 4000 (GE Healthcare Bio-Sciences Corp). Densitometric analysis of target bands at 17 kD was determined by NIH Image J software. The internal controls were used to normalize the density among multiple membranes. The tissue protein was extracted as previously described, and 20 μg of total tissue protein was used to quantify ileum tissue lysozyme.

Quantitative Cryptdin 4 PCR Analysis

RNA was extracted from frozen distal ileal tissue using the SV Total RNA isolation kit (V3100; Promega, Madison, WI), according to the manufacturer's protocol. The RNA purity

and concentration was determined by Nanodrop (Thermo Fisher Scientific, Hanover Park, IL), and 1 μg of RNA was used as a template for reverse transcription using random primers and ImProm-II reverse transcriptase in a complementary DNA (cDNA) synthesis reaction according to the ImProm-II Reverse transcription kit's instructions (cat. A3800; Promega). Quantitative real-time polymerase chain reaction (qPCR) was performed using the SYBRSelect Master MixSystem (Applied Biosystems, Foster City, CA). The primers for cryptdin 4, including forward primer (5′-CCAGGCTGATCCTATCCAAA-3′) and reverse primer (5′-ATTCCACAAGTCCCACGAAC-3′), were designed and composed by Invitrogen (Grand Island, NY). The cryptdin 4 mRNA expression level was determined using 7500 fast real-time PCR system software (Applied Biosystems, Foster City, CA) with a ΔCT relative quantification model. The geometric mean of the expression levels of the reference gene, β-actin, was calculated and used as a normalization factor.

Luminal Cytokine Quantitative Analysis

Concentrations of IL-4, IL-13, and IL-10 were determined in the small intestinal wash fluid using solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA), according to the manufacturer's instructions as described in our previous work.[7,22] The V_{max} Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA) was used to measure the absorbance values of samples at 450 nm. The cytokine concentrations in the samples were calculated by using a 4-parameter logistic fit standard curve (SOFTmax PRO software; Molecular Devices).

Immunohistochemistry

The fixed ileal tissue sections were processed (Tissue-Tek V.I.P.; Sakura Finetek) and embedded in paraffin. Sections were cut 5 μm thick and deparaffinized. Antigen retrieval was in sodium citrate buffer (Dako REAL Target Retrieval Solution; DakoCytomation, Carpinteria, CA). Sections were blocked in 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour and incubated with primary rabbit anti–human lysozyme (A0099; DakoCytomation) with 1:1000 dilution in 1% BSA-PBS overnight at 4°C. Next, the goat anti–rabbit IgG-TR (SC 2780; Santa Cruz Biotechnology) with 1:400 dilution in 1% BSA-PBS was applied as an appropriate secondary antibody for 30 minutes at room temperature. Nuclei were counterstained with DAPI (cat. P36935; Invitrogen). The immunohistochemistry (IHC) features shown are representative of all tissue samples studied.

Bacterial Preparation

The ampicillin-resistant *Escherichia coli* 5011-Lux was prepared 72 hours prior to the day of the EVISC experiment. It was cultured in 40 mL tryptose broth (LB, 100 μg/mL ampicillin) for 48 hours at 37°C under 5% CO_2. Then, 1 mL of the aliquot from the surface was transferred to a new 40-mL LB broth containing 100 μg/mL ampicillin for 24 hours at 37°C under 5% CO_2. Next, the culture was centrifuged at 1780 *g* for 11 minutes to obtain the pellet and the pellet was resuspended in 40 mL LB to wash twice. Finally, it was resuspended in 1 mL Dulbecco's phosphate-buffered saline (DPBS) to obtain a bacteria stock solution. The bacteria stock solution was diluted at a ratio of 1:100 to DPBS, and the value was measured on a spectrophotometer (DU640B; Beckman, Brea, CA) at a 450-nm wavelength. Bacterial concentrations were calculated based on previously established growth curves and adjusted to 1×10^8 colony-forming units (CFU)/mL.
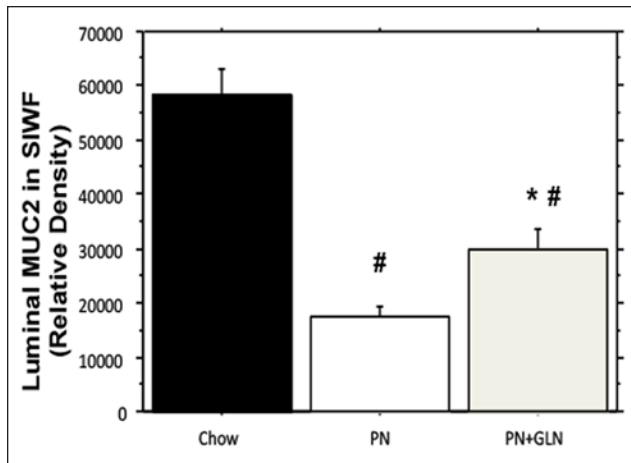
EVISC

The EVISC method was performed as previously described.[34] Briefly, 2 cm of distal ileum tissue were opened apical side up and kept hydrated in RPMI. One side of a plastic tissue disk (9 mm outer aperture and 6 mm internal aperture) was applied lightly with tissue glue (Dermabond; Ethicon, Cornelia, GA) and placed onto the mucosal side of intestinal tissue. Another tissue disk was glued on the serosal side of the intestinal segment. After covering the bottom of the serosal disk with a light layer of tissue glue, the intestinal segment was set into the bottom of a cell culture insert (cat. 3180, 0.4 μM pore, 12-well format; BD Bioscience, Franklin Lakes, NJ) with gentle pressure.

Then, 400 μL of bacterial solution (1×10^8 CFU/mL) in RPMI containing ampicillin (100 μg/mL) was placed into the wells for 1 hour at 37°C. After washing, gentamicin (100 μg/mL) was added for 1 hour at 37°C to kill remaining bacteria in the well or adherent to the mucosal surface. RPMI containing gentamicin was removed and washed. Next, 400 μL of 0.1% Triton-X in DPBS was added to each well, and the plates were agitated on an orbital shaker (175 rpm; Classic Series C1 Shaker; New Brunswick Scientific, Enfield, CT) for 30 minutes at room temperature. Serial dilutions (10^1–10^7) of the cell lysate in DPBS were plated on LB agar plates containing ampicillin (100 μg/mL) and incubated for 18 hours at 37°C. Enteroinvasion was assessed by counting CFU of ExPEC grown on the plates.

Statistical Analysis

All the data are reported as means ± standard error of mean (SEM). A fixed-effects analysis of variance (ANOVA) model with the Fisher protected least significant difference post hoc test (PLSD) was chosen for determining the statistical significance. All statistical calculations were performed with Stat View software (Abacus Concepts, Berkeley, CA). Statistical significance was accepted at $P < .05$.

Figure 1. Morphological changes after feeding chow, parenteral nutrition (PN), and PN + glutamine (GLN). (A) Goblet cells per villi in mice. (B) Representative images of periodic acid–Schiff (PAS) base-stained ileum tissue are shown for chow, PN, and PN + GLN. PAS-stained goblet cells are visible in the epithelial layer of the villus and crypts (original magnification, ×20).

Results

Analysis of Goblet Cell Number

PN significantly reduced the number of goblet cells per villi compared with chow (chow, 14.0 ± 0.70 vs PN, 8.74 ± 0.35; $P < .0001$). The addition of GLN to PN significantly increased the number of goblet cells per villi compared with PN alone (PN + GLN, 13.4 ± 0.59 vs PN, 8.74 ± 0.35; $P < .0001$), to levels similar to chow (chow, 14.0 ± 0.70 vs PN + GLN, 13.4 ± 0.59; $P = .5$) (Figure 1A). The average cell numbers per group are shown (Figure 1B).

Analysis of Intestinal Fluid MUC-2

PN significantly lowered the relative density of intestinal fluid MUC2 compared with chow mice (chow, $58,244 \pm 4700$ vs PN, $17,400 \pm 1921$; $P < .0001$). The addition of GLN

to PN significantly increased the luminal level of MUC2 in the SIWF compared with PN alone (PN + GLN, $29,771 \pm 3701$ vs PN, $17,400 \pm 1921$; $P = .01$); however, the level of MUC2 remained significantly lower than that in chow (PN + GLN, $29,771 \pm 3701$ vs chow, $58,244 \pm 4700$; $P < .0001$) (Figure 2).

Analysis of Intestinal Fluid Lysozyme

PN significantly decreased lysozyme (relative density) in the intestinal fluid compared with chow (PN, 293 ± 88.7 vs chow, 5742 ± 813; $P < .0001$). The addition of GLN to PN significantly increased lysozyme levels in the intestinal fluid compared with PN alone (PN + GLN, 1507 ± 108 vs PN, 293 ± 88.7; $P = .04$), but lysozyme levels remained significantly lower than chow (PN + GLN, 1507 ± 108 vs chow, 5742 ± 813; $P < .0001$) (Figure 3).

Figure 2. Relative density of mucin 2 (MUC2) from the small intestinal wash fluid (SIWF). Data are presented as mean ± SEM. #$P < .0001$ vs chow. *$P = .01$ vs parenteral nutrition (PN).
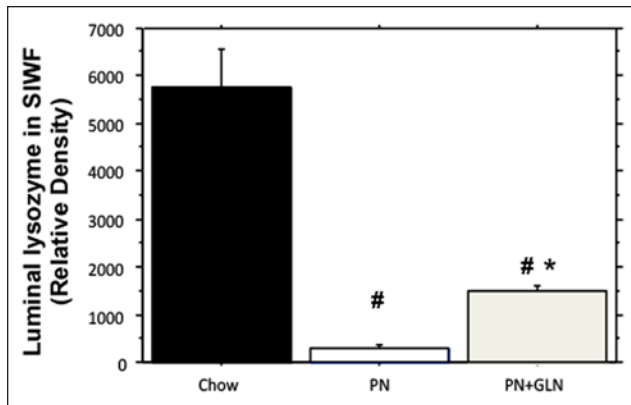
Figure 3. Relative density analysis of lysozyme from the small intestinal wash fluid (SIWF). Data are presented as mean ± SEM. #$P < .0001$ vs chow. *$P < .05$ vs parenteral nutrition (PN). GLN, glutamine.

Analysis of Small Intestinal Wash Levels of IL-4, IL-13, and IL-10

IL-4 and IL-13 levels in the small intestinal washes of the PN group were significantly lower than those in the chow group ($P = .02$ and $P < .05$, respectively) (Table 1). Both levels in the PN + GLN group were significantly higher than those in the PN group ($P = .02$ and $P = .005$, respectively). There were no significant differences in IL-4 and IL-13 levels between the chow and PN + GLN groups (IL-4, $P = .9$; IL-13, $P = .4$). PN significantly reduced IL-10 in the small intestinal washes compared with chow ($P = .0008$). The addition of GLN to PN significantly increased the luminal levels of IL-10 compared with PN alone ($P = .007$), and IL-10 was statistically similar to the chow group ($P = .3$) (Table 1).
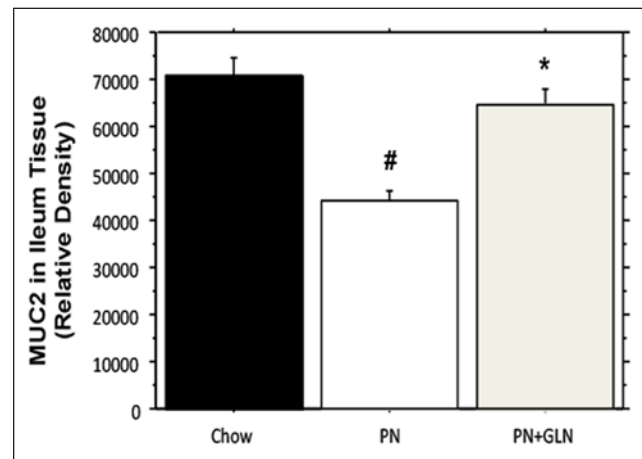
Table 1. Luminal IL-4, IL-13, and IL-10 Concentrations in Small Intestinal Wash Fluid.

Cytokines	Chow	PN	PN + GLN
IL-4, pg/mL	155.7 ± 15.3	109.3 ± 9.6[a]	157.7 ± 12.6[b]
IL-13, pg/mL	8.6 ± 0.5	5.6 ± 1.0[a]	9.7 ± 1.1[b]
IL-10, pg/mL	1366.5 ± 114.1	812.8 ± 76.3[a]	1216.0 ± 107.2[b]

Data are presented as mean ± SEM. GLN, glutamine; IL, interleukin; PN, parenteral nutrition.
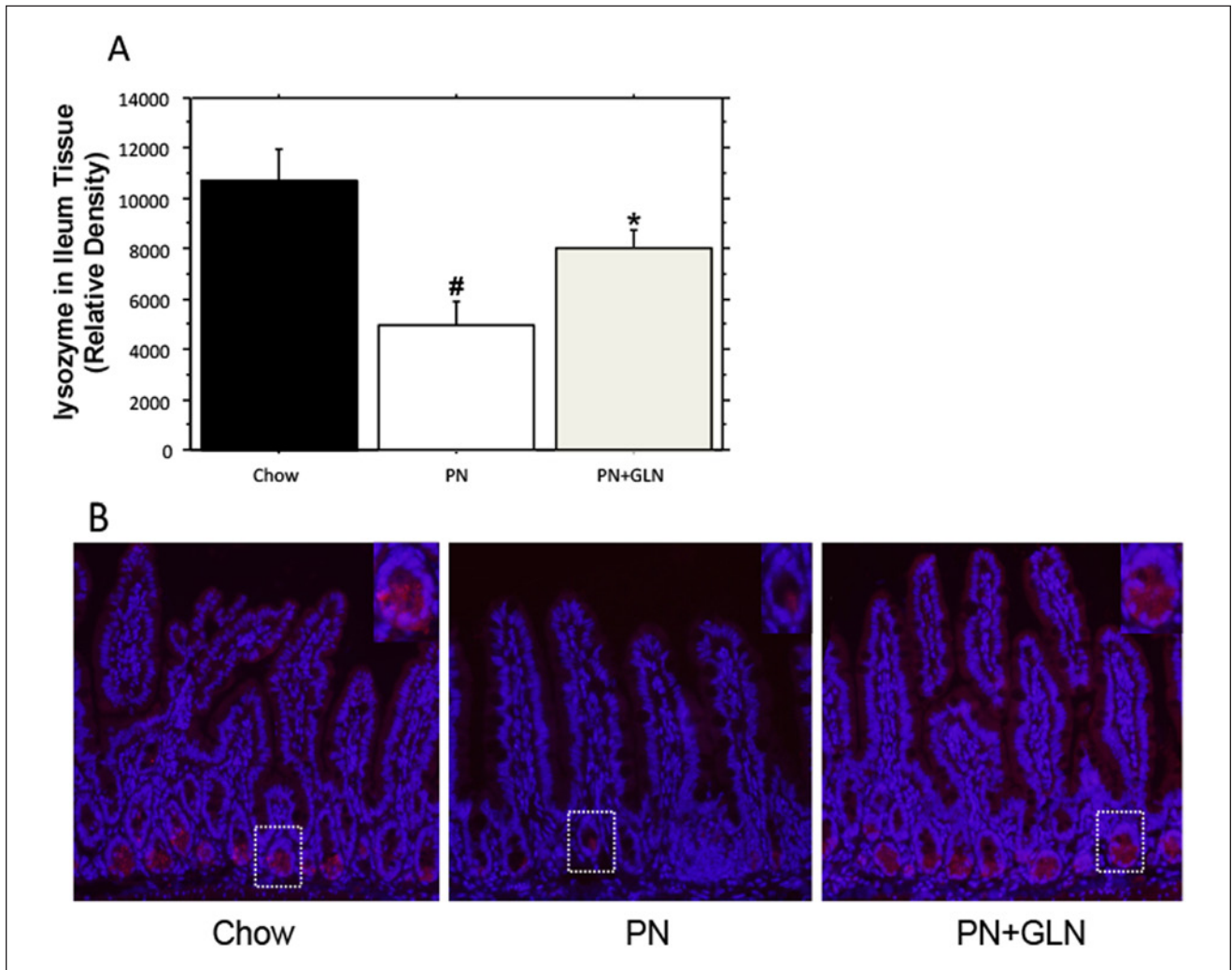[a]$P < .05$ vs chow.
[b]$P < .01$ vs PN.

Figure 4. Relative density of mucin 2 (MUC2) in ileum tissue. Data are presented as mean ± SEM. #$P < .0001$ vs chow. *$P < .0001$ vs parenteral nutrition (PN). GLN, glutamine.

Analysis of Ileum Tissue MUC-2

Consistent with the change of goblet cell numbers, the relative density of ileum tissue MUC2 was significantly decreased in the PN-alone group compared with that in chow mice (chow, 70,762 ± 3844 vs PN, 44,008 ± 2385; $P < .0001$). The addition of GLN to PN significantly increased the tissue level of MUC2 compared with PN alone (PN, 44,008 ± 2385 vs PN + GLN, 64,565 ± 3484; $P < .0001$), and the MUC2 relative level was statistically similar to the chow group (chow, 70,762 ± 3844 vs PN + GLN, 64,565 ± 3484; $P = .2$) (Figure 4).

Analysis of Ileum Tissue Lysozyme

PN significantly decreased the ileum tissue lysozyme (relative density) compared with chow (PN, 4977 ± 936 vs chow, 10,717 ± 1206; $P = .0002$). The addition of GLN to PN significantly enhanced the tissue level of lysozyme compared with PN alone (PN, 4977 ± 936 vs PN + GLN, 8051 ± 687; $P = .02$), and the level of tissue lysozyme remained similar to the chow group (PN + GLN, 8051 ± 687 vs chow, 10,717 ± 1206; $P > .05$) (Figure 5A). IHC for lysozyme in the ileum showed that PN

Figure 5. Relative density of lysozyme in ileum tissue. (A) Ileal tissue lysozyme levels. Data are presented as mean ± SEM. #*P* < .0001 vs chow. *P* < .0001 vs parenteral nutrition (PN). (B) Representative immunohistochemistry for lysozyme in ileum tissue from chow (left panel), PN (middle panel), and PN + glutamine (GLN) (right panel). Original magnification, ×20. Insets: Magnifications of single crypts from the 3 groups (original magnification, ×40). The histologic features are representative for all tissue samples studied.

reduced the expression of lysozyme compared with chow. The overall expression of lysozyme was increased after 5 days of GLN supplementation to PN (Figure 5B).
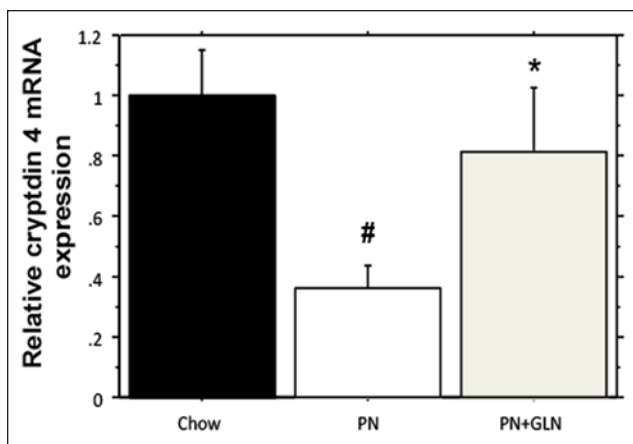
Analysis of Ileum Cryptdin 4 mRNA Expression

qPCR analysis of cryptdin 4 showed relative mRNA expression was significantly depressed (chow, 1.0 ± 0.15 vs PN, 0.36 ± 0.08; *P* = .006) after PN compared with chow. GLN supplementation to PN significantly increased cryptdin 4 mRNA expression compared with PN alone (PN + GLN, 0.81 ± 0.21 vs PN, 0.36 ± 0.08; *P* = .039), and the cryptdin 4 mRNA expression

was not significantly different from the chow group (chow, 1.0 ± 0.15 vs PN + GLN, 0.81 ± 0.21; *P* = .4) (Figure 6).

Bacterial (ExPEC) Invasion After EVISC

Compared with chow, the number of invaded *E coli* recovered from intestinal explants significantly increased following PN (chow, 457,462.500 ± 65,679.411 vs PN, 919,800.000 ± 158,808.832; *P* = .01). The addition of GLN to PN significantly reduced the number of invaded *E coli* compared with PN alone (PN, 919,800.000 ± 158,808.832 vs PN + GLN, 556,200.000 ± 118,085.619; *P* = .04). The number of invaded *E coli* in the GLN group was limited to levels observed in

Figure 6. Quantitative polymerasr chain reaction analysis of cryptdin 4 expression in ileum tissue. [#]$P < .01$ vs chow. [*]$P < .04$ vs parenteral nutrition (PN). GLN, glutamine; mRNA, messenger RNA.
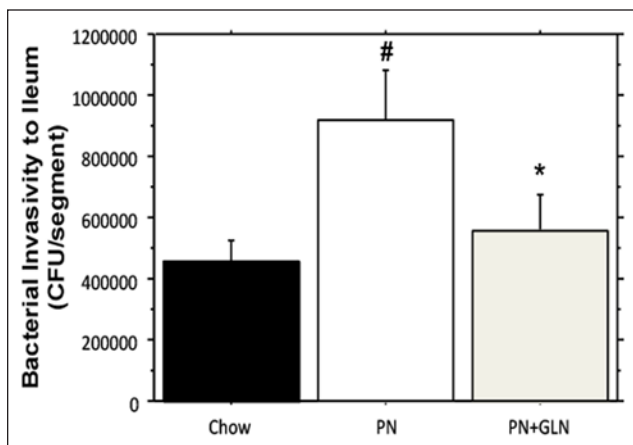
Figure 7. Effect of chow, parenteral nutrition (PN), and PN + glutamine (GLN) feeding on susceptibility of ileal tissue to invasion by bacteria. Data are presented as mean ± SEM. [#]$P < .05$ vs chow. [*]$P < .05$ vs PN.

chow (chow, 457,462.500 ± 65,679.411 vs PN + GLN, 556,200.000 ± 118,085.619; $P > .05$) (Figure 7).

Discussion

Several clinical studies reported that perioperative GLN-PN reduces the morbidity from infectious complications in patients undergoing abdominal surgery or trauma compared with the standard PN.[35-37] Our work has established that PN impairs mucosal immunity, providing a cogent explanation for this vulnerability. The mucosal immune system comprises both innate and adaptive immune arms. Both systems respond to potentially harmful antigens within the intestinal lumen, including environmental, bacterial, viral, and dietary antigens. The adaptive immune system

consists of specialized T and B lymphocytes, macrophages, and dendritic cells that coordinate production and transport of sIgA from the lamina propria onto mucosal surfaces. Our prior work demonstrated that GLN supplementation improved adaptive immunity lost with PN feeding alone through several mechanisms.[8,22] Experimentally, GLN added to PN improved survival in immunized mice after intratracheal administration of *Pseudomonas aeruginosa* compared with PN alone through maintenance of sIgA in the upper respiratory tract.[6,37] The second arm of mucosal immunity—innate immunity—provides nonspecific, immediate, and continuous defense against bacterial invasion of epithelial surfaces. To our knowledge, this work is the first investigation of GLN effects on this more teleologically ancient line of host defense.

Innate immunity consists in part of specialized secretory epithelial cells, including goblet and Paneth cells, which reside within the mucosal epithelial layer and respond to pathogens through secretion of a physicochemical mucous barrier. These secretions consist of a complex fluid rich in mucin glycoproteins that concentrate a wide variety of antimicrobial peptides released from the Paneth cells as well as the sIgA transported from the lamina propria. In the small intestine, this layer covers the epithelium and helps prevent enteric pathogens from reaching the underlying epithelial layer. GLN maintains several aspects of intestinal defense compared with PN alone. The most abundant mucin glycoprotein in this extracellular mucus barrier is MUC2 and, consistent with our previous studies, PN significantly decreases the luminal and tissue levels of MUC2.[33] In this work, PN significantly decreased the absolute number of goblet cells per villi in the small intestinal epithelium, suggesting one reason for the loss of luminal and tissue MUC2. These goblet cell number and mucus changes mirror the studies by Khan et al[38] and Ekelund et al,[39] although the latter examined jejunum. In the present work, GLN maintained the number of goblet cells per ileum villi and normalized the ileal luminal and tissue homogenate levels of MUC2 compared with PN alone, findings consistent with Khan et al, in which PN was supplemented with alanyl-glutamine. Conour et al,[40] however, used a model of PN in neonatal piglets and demonstrated goblet cell expansion in PN compared with the EN control group. In contrast to the present study, an important factor in the work by Conour et al was that the enteral control group received formula milk replacer. This constitutes a diet considerably different in complexity and composition than our control group, which was fed standard rodent chow. Furthermore, compared with adult animals, neonatal animals have considerably different adaptive immune cell compartments, so differences are not surprising between neonatal and adult animals.

Paneth cells reside at the base of each small intestinal crypt. These cells secrete a battery of antimicrobial products, including defensins, lysozyme, and sPLA$_2$, that form an immunologic defense against luminal pathogens. Enteric α-defensins released from murine Paneth cells were named cryptdins ("crypt defensins"). Cryptdin 4 is an important active form that exhibits a broad

spectrum of bacterial activities and has the greatest antimicrobial activity of the described cryptdins.[15-17,41] Cryptdin 4 eliminates gram-positive bacteria by binding lipid II and inhibiting bacterial cell wall biosynthesis and attacks gram-negative bacteria by forming membrane pores leading to bacterial lysis. In concert with cryptdin 4, another Paneth product, lysozyme, assaults gram-positive bacteria through hydrolyzing the b(1-4) glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid to induce bacterial lysis. Lysozyme functions most effectively when combined with cryptdin 4 against gram-negative bacteria since cryptdin 4 helps degrade the outer membrane of bacteria. Together, the combined activity prevents the attachment of luminal bacteria and reduces the invasion of bacteria into the intestinal mucous layer. In our study, PN significantly reduced the cryptdin 4 mRNA expression as well as both the luminal and tissue protein levels of lysozyme, consistent with our hypothesis that PN compromises Paneth cell function. Paneth cells constitute a comparably long-lived population of epithelial cells (~21 days). In our model, we observed no evidence of decreased Paneth cell numbers during the 5 days of PN feeding but only a decrease in the level of intracellular Paneth cell granules during PN.[42] Functionally, others showed that Paneth cell dysfunction results in intestinal inflammation and may lead to lethal colitis in mice.[18] Furthermore, our study reveals that GLN partially restores the protein expression of lysozyme in SIWF and completely restores tissue levels of lysozme in mouse ileum. Together, these data support the hypothesis that GLN upregulates innate immunity during PN.

Since we demonstrated improvements in innate immunity and adaptive immunity following GLN compared with PN alone, we further hypothesized that GLN would provide protective effects on barrier function. In prior work, we developed an ex vivo intestinal segment culture that simulates host-pathogen interactions occurring at the mucosal surface.[34] This model demonstrated that PN without enteral stimulation significantly increases the susceptibility of murine ileum to enteroinvasion by an extraintestinal pathogenic *E coli* isolated from a patient with recurrent urinary tract infections resistant to therapy. The results demonstrate that GLN significantly attenuates susceptibility of ileal segments to enteroinvasive *E coli* compared with PN alone to levels observed in chow. This finding is consistent with enhanced innate immune expression of cryptdin 4 and levels of lysozyme and MUC2 observed in vivo.[22] Cryptdins represented nearly 70% of secreted bactericidal compounds from Paneth cells.[18,43] Interestingly, the exogenous supplementation of glutamine failed to increase luminal sPLA$_2$ activity in this study (data not shown), a compound with important antimicrobial functions. Our prior work demonstrated PN significantly reduced sPLA$_2$ within the intestinal lumen. This suggests that not all antimicrobial compounds produced by Paneth cells are stimulated by GLN and that improvement in barrier function is not dependent on increases in all antibacterial molecules.

On a side note, we previously reported that PN significantly decreased the luminal and tissue levels of Th2 cytokines, and the addition of GLN to PN significantly improved IL-4 in small intestine homogenates and partly restored IL-10 levels compared with PN alone.[22,24] In this work, we found that adding GLN to PN completely restored levels of IL-4, IL-10, and IL-13 in the small bowel wash fluid to levels found in chow mice. We speculated in prior work that luminal cytokines could act as paracrine signals to stimulate the epithelium, affecting the epithelial cell physiology and function. These signals may have been restored with GLN, since this compound provides fuel for rapidly dividing cells (particularly lymphocytes and enterocytes) and intestinal epithelial cells.[44-46] The implications of these finding remain unclear but are a focus of further investigations in our laboratory.

In conclusion, this work supports our hypothesis that PN without EN feeding decreases Paneth cell function, through decreased lysozyme and cryptdin 4 expression, and number and function of goblet cells. Taken together, these changes impair innate immunity and increase susceptibility of the mucosa to bacterial invasion. GLN supplementation improves innate immunity by improving Paneth and goblet cell parameters, normalizing Th2 cytokines, and increasing resistance to bacterial mucosal invasion. This study confirms the importance of enteral feeding upon innate immunity as well as the potential importance of providing parenteral nutrient supplements for the protection of the intestinal mucosa.

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