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Parenteral Nutrition Impairs Lymphotoxin β Receptor Signaling via NF- κ B

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Structured Abstract

Objective—To determine effects of 1) parenteral nutrition (PN) 2) exogenous Lymphotoxin β receptor (LT β R) stimulation in PN animals and 3) exogenous LT β R blockade in chow animals on NF- κ B activation pathways and products: MAdCAM-1, chemokine (C-C motif) Ligand (CCL) 19, CCL20, CCL25, interleukin (IL)-4 and IL-10.

Summary Background Data—Lymphotoxin (LT) stimulates LT β R in Peyer's patches (PP) to activate NF- κ B via the non-canonical pathway. The p100/RelB precursor yields p52/RelB producing MAdCAM-1, cytokines, and chemokines important in cell trafficking. TNF α , IL-1 β , & bacterial products stimulate the inflammatory canonical NF- κ B pathway producing p65/p50 and c-Rel/p50. PN decreases LT β R, MAdCAM-1 and chemokines in PP and lowers small intestinal (SI) IgA compared to chow.

Methods—Canonical (p50 & p65) and non-canonical (p52 & Rel B) NF- κ B proteins in PP were analyzed by TransAM NF- κ B kit after 5 days of chow or PN, 2 days of LT β R stimulation or 3 days of LT β R blockade. MAdCAM-1, chemokines and cytokines in PP were measured by ELISA after LT β R stimulation or blockade.

Results—PN significantly reduced all NF- κ B proteins in PP compared to chow. Exogenous LT β R stimulation during PN increased p50, p52, Rel B, MAdCAM-1, IL-4 and IL-10 in PP, but not p65, CCL19, CCL20 or CCL25 compared to PN. LT β R blockade reduced non-canonical products (p52 and Rel B), MAdCAM-1, CCL19, CCL20, CCL25, IL-4 and IL-10 but had no effect on the inflammatory pathway (p50 and p65) compared to chow.

Conclusion—Lack of enteral stimulation during PN decreases both canonical and non-canonical NF- κ B pathways in PP. LT β R stimulation during PN feeding completely restores PP non-canonical NF- κ B activity, MAdCAM-1, IL-4, IL-10, and partly the canonical pathway. LT β R blockade decreases the non-canonical NF- κ B activity, MAdCAM-1, chemokines and cytokines without effect on the canonical NF- κ B activity in PP.

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Introduction

The mucosal associated lymphoid tissue (MALT) provides the specific immune protection of moist mucosal surfaces and constitutes the largest immune organ outside the liver and spleen. It produces IgA to protect against the huge load of bacteria and toxins within the gut lumen as well as the normally sterile pulmonary tissue. Naïve T&B cells destined for mucosal immunity express two integrins - L-selectin and $\alpha_4\beta_7$ - directing them into Peyer's patches (PP) of the small intestine via interaction with mucosal addressin cellular adhesion molecule-1 (MAdCAM-1), a molecule expressed on the high endothelial venules of the Peyer's patches. 1·2 Regional chemokines stimulate migration of the cells into the Peyer's patches: CCL19 regulates T-cell entry, CCL-25 recruits antibody-secreting cells, and CCL-20 recruits dendritic cells.3 The T&B cells are sensitized in PP to luminal antigens absorbed by specialized M cells covering the PP.4 Our previous work shows that blockade of either the integrins- L-selectin and $\alpha_4\beta_7$ - or the adhesion molecule- MAdCAM-1 - reduces cell entry into MALT, reduces secretory IgA and impair of mucosal immune protection.1·2 This work examines the specific pathways involved in this entry.

Lymphotoxin β receptor (LT β R) expressed in the endothelial surfaces of PPs control production of nuclear factor-kappa B (NF- κ B) which is the key transcriptional regulator of these critical entry molecules *in vitro*. Normally, lymphotoxin α and β (LT α 1 β 2) expressed on activated T&B cells stimulates the lymphotoxin β receptor (LT β R).5 This interaction activates NF- κ B via the non-canonical pathway to produce MAdCAM-1, cytokines, and the specific chemokines critical to cell entry and function3. LT β R deficient (LT β R $-/-$) mice lack PP and lymph nodes and express low levels of MAdCAM-1, CCL-20, CCL25, and CXCL13. These mice also have lower levels of two Th-2 type IgA-stimulating cytokines, IL-4 & IL-10, and IgA itself. We and others showed that administration of a fusion protein which blocks LT β R interfering with this LT β R/ lymphotoxin interaction reduced the size and numbers of lymphocytes in PP. 5·6.

PN also interferes with this sequence. Our previous work3·7·9 demonstrated that PN feeding decreases the number of PP lymphocytes and the mRNA or/and protein levels of MAdCAM-1, CCL-20, CCL25, CXCL13 as well as LT β R itself compared to chow fed mice. The administration of a stimulatory (agonistic) LT β R antibody to PN mice prevented the reduction in PP lymphocytes and intestinal IgA.7 Also, LT β R blockade in chow fed animals significantly reduced MAdCAM-1 mRNA level and PP lymphocyte mass. 6 In this work we hypothesized that the PN effects occurs through down-regulation of NF- κ B pathway(s). Two distinct NF- κ B activation pathways have been described (Figure 1).10 The canonical (classical) NF- κ B pathway is activated by proinflammatory cytokines, toll like receptor (TLR) ligands, or stresses such as γ -radiation, ultraviolet light and oxidative stress. These stimuli induce degradation of I κ B α and the nuclear accumulation of mainly p50-RelA (p65) dimer, which regulate the expression of immunoregulatory and anti-apoptotic genes but not the cell entry proteins. The pathway which we hypothesize is relevant to the PN changes is the non-canonical (alternative) NF- κ B pathway which is activated by receptors involved in lymphoid organogenesis and lymphocyte development. Receptors such as LT β R induce processing of p100 to p52 and nuclear accumulation of p52-RelB dimers. This non-canonical NF- κ B pathway appears to control processes directing PP T&B cell entry. In this work, we define in detail the mechanisms of PN-induced immune deficits and hypothesize that PN negatively affects LT β R signaling via the non-canonical NF- κ B pathway. To test this hypothesis, we quantified the inflammatory canonical NF- κ B pathway products - p50 & p65- as well as the non-canonical NF- κ B pathway products - p52 and Rel B levels - together with levels of MAdCAM-1, CCL-19, CCL20, CCL25, IL-4 and IL-10 in the PP of animals receiving PN or chow feeding. To establish cause and effect, we measured

these same parameters in PN fed animals administrated an agonistic LT β R mAb and chow fed animals receiving LT β R blockade.

Methods

Animals

All experimental protocols were approved by the Animal Care and Use Committee of the University of Wisconsin–Madison and Middleton Veterans Administration Hospital, Madison, WI. Six-week-old, male mice from Institute of Cancer Research (Harlan, Indianapolis, IN) were used for the experiments. The mice were kept in an environment with controlled temperature, humidity, and light cycle (12-hour light/12-hour dark). They were acclimatized for 1 week and fed a standard mouse chow (PMI Nutrition International, St. Louis, MO) ad libitum before protocol entry. During feeding protocol, mice had free access to water and were individually housed in metal metabolism cages with wire grid floors to eliminate coprophagia.

Experiment 1: Effects of PN on NF- κ B activation pathways

Mice received catheters for IV infusion after intraperitoneal injection of a mixture of ketamine (100 mg/kg body weight) and acepromazine maleate (5 mg/kg body weight). A silicone rubber catheter (0.012" I.D. by 0.025" O.D.; Helix Medical, Inc., Carpinteria, CA) was inserted into the vena cava through the right jugular vein. The distal end of the catheter was tunneled subcutaneously and exited the midpoint of the tail. Mice were partially immobilized by tail restraint to protect the catheter during infusion. This technique of infusion in the mouse has been demonstrated to be an acceptable method of nutritional support, and this method does not produce physical or biochemical evidence of stress⁸.

Thirty-five mice were randomized to receive chow (Chow, n = 19) or parenteral nutrition (PN, n = 16). Mice received 0.9% saline at a rate of 4 mL/d with free access to water and chow for 2 days after surgery. PN mice initially received 4 mL/day of PN and advanced to a goal rate of 10 mL/day by the third day. The PN solution contains 6.0% amino acids, 34.9% dextrose (6002 kJ/L), electrolytes, and multivitamins, with a nonprotein calorie/nitrogen ratio of 535.8 kJ/g nitrogen. This feeding meets the calculated nutrient requirements of mice weighing 25 to 30g. 11

After 5 days of dietary treatments, animals were anesthetized and exsanguinated by cardiac puncture. The small intestine was removed, and the mesenteric fat and external vasculature were dissected away. After washing with 20 mL cold calcium and magnesium-free Hanks balanced salt solution (HBSS, BioWhittaker, Walkersville, MD), the PP were removed from the serosal side of the intestine. The PP were frozen in liquid N₂ and stored at -80°C until the levels of Phosphorylated NF- κ B proteins were measured.

Phosphorylated NF- κ B proteins, p50, p65, p52 and Rel B in PP were analyzed by TransAM NF- κ B Family kit (Active Motif, Carlsbad, CA) after 5 days of chow or PN. TransAM™ NF- κ B Kits are DNA-binding ELISAs that facilitate the study of transcription factor activation in mammalian tissue and cell extracts. The manufacturer's protocol was followed. Briefly, protein samples from whole-cell extracts were prepared by using Nuclear Extract Kit (Active Motif, Carlsbad, CA) on ice to disrupt and homogenize PP in 1 ml ice-cold Complete Lysis Buffer with an Omni's TissueMaster Homogenizer (Fisher Scientific, Marietta, GA). Homogenization began at slow speeds until the PP were broken into smaller pieces and then the speed was increased to the maximum for 45-60 seconds. Care was taken to avoid the generation of excess heat or foam during homogenization. The homogenate was then transferred to a pre-chilled microcentrifuge tube, centrifuged at 10,000 \times g for 10 minutes at 4 °C. The supernatant was then transferred to a new pre-chilled tube

and centrifuged again. The supernatant fluid is the whole-cell lysate. The protein concentration was quantified by the dye-binding Bradford reagent (BioRad, Hercules, CA), and the samples were stored at -80°C until analyzed for NF- κB proteins.

After optimization, in each well, 5 μg of protein diluted in 20 μl Complete Lysis Buffer and 30 μl binding buffer were incubated at room temperature for 1 h in a 96-well plate coated with an oligonucleotide that contained the NF- κB consensus binding site under mild agitation. Then, the plate was washed. p65, p50, p52 or RelB antibodies were added to the wells. The plate was incubated for 1 h at room temperature, and then it was washed. Following this step, the secondary antibody was added to the wells. The plate was incubated for 1 h at room temperature, and then washed. Finally, the developing solution was added. After 5–10 min incubation at room temperature, the stop solution was added and the absorbance was read at 450 nm using a microplate reader (Molecular Devices Corporation, CA).

Experiment 2: Effects of LT βR Stimulation on NF- κB activities, MAdCAM-1, Chemokines and Cytokines

Mice received IV catheters as described in experiment 1. Two days after cannulation, 48 mice were randomized to Chow ($n = 13$), PN plus agonistic LT βR mAb (PN-LT βR , $n = 17$), or PN plus isotype control antibody (PN-Ig; $n = 18$). Chow and PN diets were identical to experiment 1. Using the previously optimized dose for the LT βR agonistic antibody obtained from dose response experiments show in our prior work 7, Chow mice received 200 μL of vehicle phosphate buffered saline (PBS), twice a day for 2 days. PN-LT βR and PN-Ig groups received the same volume containing 5 μg of hamster agonistic LT βR mAb (a generous gift from Jeffrey L. Browning, Biogen Idec, 12 Cambridge Center, Cambridge, MA) or 5 μg of hamster IgG1 κ (Becton Dickinson Pharmingen, San Diego, CA), respectively, twice a day for 2 days.

After 2 days of treatment, mice were exsanguinated under anesthesia. The small intestine was excised from the duodenal bulb to ileocecal valve and the lumen flushed with 20 mL of HBSS. The PP were removed from the serosal side of the intestine then frozen in liquid N_2 and stored at -80°C . Phosphorylated NF- κB proteins in PP were analyzed as Experiment 1.

Commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits were purchased for MAdCAM-1, CCL19, CCL20, CCL25 (R&D Systems, Minneapolis, MN), IL-4 and IL-10 (Becton Dickinson Pharmingen, San Diego, CA). Ninety-six-well plates were coated with capture antibody diluted to working dilution in phosphate-buffered saline (PBS) and incubated overnight at room temperature (RT) in a humid chamber. The following morning, plates were washed 3 times with PBS + 0.05% Tween 20 (PBS-T), and nonspecific binding was blocked by Reagent Diluent (R&D Systems, Minneapolis, MN) for 1 hour. Plates were washed 3 times before addition of samples. Samples (100 μL /well) were loaded at 80 $\mu\text{g}/\text{mL}$ protein diluted with Reagent Diluent and incubated for 2 hours at RT. Plates were washed 3 times with PBS-T before addition of the detection antibody (100 μL /well) diluted 1:500 in Reagent Diluent and incubated at RT for 1 hour. Plates were washed 3 times before addition (100 μL /well) of the IgG-HRP conjugate at a dilution of 1:20,000 in Reagent Diluent. The conjugate was incubated at RT for 1 hour. Tetramethyl benzidine (TMB; BD Biosciences, San Diego, CA; 100 μL /well) substrate was added and incubated at RT for 15 minutes before the reaction was stopped with 50 μL /well of 2-N H_2SO_4 . The absorbance was read at 450 nm using a microplate reader (Molecular Devices Corporation, CA).

Experiment 3: Effects of LT β R Blockade on NF- κ B activities, MAdCAM-1, Chemokines and Cytokines

Twenty-seven mice received central venous catheters as described in Experiment 1. After recovering from surgery for 2 days, the animals were then randomly assigned to Chow-LT β R-Immunoglobulin (Chow- anti LT β R) group (n=10), Chow + control immunoglobulin (Ig) group (Chow-Ig n=9) and PN + control Ig group (PN-Ig, n=8). Using our previously optimized dose noted in our prior experiments 6, Chow- anti LT β R mice received 100 μ g LT β R-Ig fusion protein IV (anti LT β R, 100 μ g in 250 μ L PBS; a generous gift from Yang-Xin Fu, Chicago University, Chicago, IL), while Chow- Ig and PN-Ig groups received human IgG IV (100 μ g in 250 μ L PBS; Sigma, St. Louis, MO). The Chow- anti LT β R treated mice continued with chow ad libitum and 0.9% saline at 4 mL/d IV throughout the study. The control Ig-treated mice were randomly assigned to either chow or PN. The PN mice initially received 4 mL/d of PN and were advanced to a goal rate of 10 mL/d by the third day of feeding. After 3 days of these dietary treatments, the animals were anesthetized with a ketamine/acepromazine mixture and exsanguinated by cardiac puncture. The small intestine was excised from the pylorus to terminal ileum and the mesenteric fat and external vasculature were dissected away. Then, the intestinal lumen was flushed by 20 mL calcium- and magnesium-free Hanks' balanced salt solution (HBSS). The PP were removed from the intestine, frozen in liquid N₂ and stored at -80°C until used in our assay. Phosphorylated NF- κ B proteins in PP were analyzed as Experiment 1 while MAdCAM-1, CCL19, CCL20, CCL25, IL-4 and IL-10 were assayed as Experiment 2.

Statistical Analyses

All values were expressed as mean \pm SD. Statistical analysis was performed with Statview software, by analysis of variance (ANOVA), followed by the Fisher's protected least significant difference post hoc test.

Results

Experiment 1: Effects of PN on Phosphorylated NF- κ B in PP (Fig 2)

PN significantly reduced all Phosphorylated NF- κ B proteins in PP compared to Chow mice (Fig.2): p50 (p<0.01), p65 (p<0.01), p52 (p<0.01) and Rel B (p<0.05).

Experiment 2: Effects of LT β R Stimulation on Phosphorylated NF- κ B, MAdCAM-1, CCL19, CCL20, CCL25, IL-4 and IL-10 in PP (Fig 3&4)

Compared with PN-Ig (Fig.3), stimulation of PN mice with LT β R agonistic antibody significantly increased the NF- κ B inflammatory pathway molecule p50 (p<0.01) and the NF- κ B non-canonical pathway molecules p52 (p<0.01) and Rel B (p<0.05) in PP. There were no significant differences in levels of p65, p52 and Rel B between PN-LT β R and Chow mice, however, LT β R stimulation of PN animals failed to improve p50 to Chow levels (p<0.01). Compared to Chow feeding, PN-Ig (Fig.4) significantly lowered levels of MAdCAM-1 (p<0.05), CCL19 (p<0.01), CCL25 (p<0.01), IL-4 (p<0.05), and IL10 (p<0.01), but not CCL20. Compared to PN-Ig mice, PN-LT β R significantly increased MAdCAM-1 (p<0.01), IL-4(p<0.01) and IL-10(p<0.05) but had no effect on CCL19, CCL20 and CCL25 in PP. There were no significant differences in levels of MAdCAM-1, CCL19, CCL20, IL-4 and IL-10 between PN-LT β R and Chow mice.

Experiment 3: Effects of LT β R Blockade on Phosphorylated NF- κ B, MAdCAM-1, CCL19, CCL20, CCL25, IL-4 and IL-10 in PP (Fig 5&6)

Compared with Chow-Ig, blockade of LT β R in chow animals using the LT β R-Ig fusion protein significantly reduced the NF- κ B non-canonical pathway molecules p52 (p<0.01) and

Rel B ($p < 0.01$) but had no effect on the NF- κ B inflammatory pathway molecules p50 and p65, (Fig.5). The blockade of LT β R significantly reduced the non-canonical downstream products (Fig.6) of MAdCAM-1 ($p < 0.05$), CCL19 ($p < 0.01$), CCL20 ($p < 0.05$), CCL25 ($p < 0.05$), IL-4 ($p < 0.05$) and IL-10 ($p < 0.05$) compared to Chow-Ig.

There were no significant differences between Chow- anti LT β R and PN-Ig in levels of MAdCAM-1, CCL19, CCL20, CCL25 and IL-10. Only levels of IL-4 were higher in Chow-anti LT β R mice compared to PN ($p < 0.05$).

Discussion

Intestinal lymphoid tissues induce adaptive immunity to pathogens while maintaining a symbiotic homeostasis with commensal bacteria in the gut lumen. Our prior work established that PN (with lack of enteral stimulation) reduces MAdCAM-1 within PP resulting in fewer CD4⁺ lymphocytes in the PP and lamina propria and lowers levels of 1) two critical Th2-type IgA stimulating cytokines, IL-4 & IL-10, 2) the chemokines CCL-20, CCL25, CXCL13 and 3) sIgA levels in the gut and lung 3·8·12·13. We confirmed that MAdCAM-1 recruits lymphocytes by showing that antibody blockade of MAdCAM-1 7 (as well as L-selectin or $\alpha_4\beta_7$) reduced PP lymphocytes cell to PN levels. A unifying concept tying MAdCAM-1, cytokines, and the chemokines with lymphocyte migration is LT β R. After LT stimulation by activated T cells, LT β R regulates expression of PP MAdCAM-1 and tissue chemokine ligands necessary for lymphocyte trafficking as well as cytokine production presumably via one of the NF κ B pathways 5·10. The current work confirms that NF κ B is an integral step in the expression and production of tissue adhesion molecules and cytokines responsible for attracting T&B cells into the Peyer's patches. Stimulating (or blocking) LT β R resulted in an increase (or decrease) in MAdCAM-1 as well as several chemokines and cytokines which play critical roles in attachment, migration and stimulation of mucosal immune cells to maintain intact mucosal immunity. Surprisingly, inflammatory NF κ B pathways were also controlled by LT β R stimulation to some extent.

Migration of immune cells to lymphoid organs and to effector sites is mediated by a multistep process whereby adhesion receptors, chemokines, and their receptors determine the specificity of immune cell homing.14·16 Both MAdCAM-1 and tissue specific chemokine ligands are expressed in response to LT β R. MAdCAM-1 is an important ligand for $\alpha_4\beta_7$ integrin expressed on naive T&B cells destined for the mucosal immune system. In the adult mouse, a *modified* MAdCAM-1, especially interactive with $\alpha_4\beta_7$, is constitutively expressed on high endothelial venules of PP. An *unmodified* MAdCAM-1, especially interactive with L-selectin, is expressed in mesenteric lymph nodes, postcapillary venules of the intestinal LP, the lactating mammary gland, and in sinus-lining cells in the spleen surrounding the periarteriolar lymphocyte sheath and follicle areas14·17·18. The difference in the *modified* and *unmodified* form is important since naive T&B cells predominantly express $\alpha_4\beta_7$ (with a lesser expression of L-selectin) while cells sensitized to antigen in the PP predominantly express L-selectin (with a lowered expression of $\alpha_4\beta_7$). These specific modifications direct cell into and through the MALT from entry sites (the PP) to effector sites where IgA is produced and secreted for immune protection. Our studies with monoclonal antibody blockade of MAdCAM-1 revealed a role for this adhesion molecule in mediating lymphocyte migration into PP. In the presence of reduced MAdCAM-1 expression in the PP with PN (it is not reduced in effector sites18), antigen-specific IgA-secreting PC migration to the intestinal LP is compromised. In this study as in our previous work7, LT β R stimulation with agonistic antibody reverses the PN-induced MAdCAM-1 reduction in PP to levels equivalent to Chow fed animals while LT β R-Ig fusion protein in chow mice reduces MAdCAM-1 expression to levels equivalent to PN fed animals (Fig.5).

These results occur in parallel with reduced levels of the non-canonical NF- κ B activation pathway molecules, p52 and Rel B.

Our work supports our hypothesis that LT β R exerts its activity on these adhesion and migration products through the non-canonical NF- κ B activation pathway. The non-canonical NF- κ B pathway is activated when lymphotoxin on T and B cells binds to LT β R. This pathway only requires p52/RelB heterodimer. Gene transcription is slow and long-lasting with p52-dependent signals when compared to the inflammatory canonical NF- κ B which is almost instantaneous.²⁰ In this study, PN reduced p52 and Rel B production in parallel with MAdCAM-1, the chemokines CCL-25 and CCL-19 as well as IL-4 and IL-10. LT β R stimulation of PN mice with agonistic antibody reversed several of these including MAdCAM-1, IL-4 and IL-10 (but not the chemokines) while LT β R blockade with LT β R-Ig fusion protein in Chow mice reduced p52 and Rel B to similar levels as in PN fed animals together with MAdCAM-1, CCL-19, CCL-25, IL-4 and IL-10. The changes are somewhat circumstantial since we could not directly block or stimulate NF- κ B activation to examine the response. To our knowledge non-toxic drugs that can be administered chronically *in vivo* to block or stimulate the non-canonical NF- κ B activation pathway directly do not exist.

In addition to the critical role of MAdCAM-1 in cell entry, the other end products also exert important effects on mucosal immunity. IL-4, produced by CD4⁺ T cells²¹, regulates antibody production, hematopoiesis and inflammation, as well as the development of effector T-cell responses. IL-4 can educate dendritic cells (DC) to differentially affect T cell effector activity and acts upon DC to instruct naive T cells to express the gut-associated homing receptor CCR9, which specifically binds CCL25 in small intestine.²² IL-10 is not specific to Th2 cells or regulatory T (TReg) cells but is much more broadly expressed by cells including Th1, Th2 and Th17 cell subsets, TReg cells, CD8⁺ T cells and B cells as well as cells in the innate immune system.²³ While both IL-4 and IL-10 in PP are reduced by PN feeding, these reductions are corrected by LT β R stimulation with agonistic antibody during PN and reduced in chow fed mice after LT β R-Ig fusion protein to levels equivalent with PN fed animals. These cytokine changes are consistent with changes in the expression of p52 and Rel B, the non-canonical NF- κ B proteins in our experimental system.

The only discordance between LT β R, the non-canonical NF- κ B proteins and cell products related to the chemokine levels. While CCL-19 and CCL25 levels dropped with PN and LT β R blockade, LT β R stimulation had no effect on CCL25 and minimal effect on CCL-19. This implies that CCL-19 and CCL25 expression may still need other stimulation in addition to LT β R signaling to return levels to normal. Neither PN nor stimulation affected CCL20 while LT β R blockade had some inhibitory effect upon this molecule. This may be due to the cross compensation from different NF- κ B pathways since recent research shows that TLR-5- and LT β R -dependent epithelial CCL20 expression involves the same NF- κ B binding site but distinct NF- κ B pathways and dynamics.²⁴

The reduction in the canonical inflammatory pathway products, p50 and p65, with PN was unexpected. Our previous work experimental work established that PN provokes proinflammatory responses to stress and gut inflammation by increasing expression of ICAM-1 & p-selectin and increasing neutrophil accumulation in the gut endothelium^{25,26}, by increasing expression of pulmonary e-selectin²⁶, by promoting liver and pulmonary dysfunction after gut ischemia reperfusion²⁷, and by increasing ICAM-1, pulmonary mononuclear β 2 integrin expression after a gut ischemic insult resulting in a higher mortality²⁸. This increased systemic inflammation is consistent with clinical data on metabolic responses and the development of multiple organ failure after PN^{29,30}. But these events reflect systemic, rather than local effects which may explain the discordance. Recently we noted that both injured patients and mice respond to trauma by increasing airway IgA in,

presumably, a protective response. 31 In our animal model of controlled injury, the airway IgA response can be recreated by exogenous administration of tumor necrosis factor- α (TNF α) and eliminated by blocking TNF α through administration of an anti-TNF α monoclonal antibody 32. The increase in airway concentrations of TNF α is a local rather than a systemic response. Interestingly, the intestine also responds to injury by acutely increasing luminal levels of IgA just like the lung presumably in a similar manner. Since PN eliminates both the pulmonary and airway IgA response to injury³³, the reduction in the canonical inflammatory pathway products noted in our results may explain this PN-induced defect in the IgA response to injury.

In summary, we believe that our work confirms that defects in cell entry into the MALT induced by PN with lack of enteral feeding to support nutritional status is due to a LT β R-dependent depression in the non-canonical NF- κ B activation pathway. This results in reduced expression in the levels of adhesion molecules, cytokines and, to some extent the chemokines, responsible for adhesion and attraction of naïve T&B cells into the mucosal immune system. In addition, PN with lack of enteral stimulation affects the inflammatory pathway as well. Clinically, development and administration of non-toxic agent to stimulate LT β R may provide an avenue to reverse the negative effects of PN on mucosal immunity.

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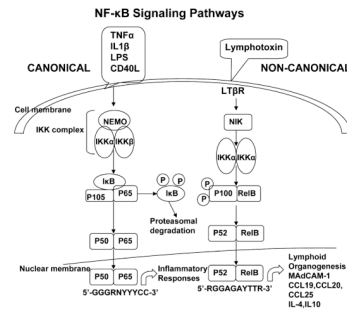


Figure 1. NF- κ B Signaling Pathways

Two distinct NF- κ B activation pathways have been described. The canonical (classical) NF- κ B pathway is activated by a large number of stimuli, including tumor necrosis factor (TNF)- α , interleukin(IL)-1 β , lipopolysaccharide(LPS), and CD40 ligand(CD40L). These stimuli induce the degradation of I κ B α and the nuclear accumulation of mainly p50–RELA (p65) dimer, which regulate the expression of immunoregulatory and anti-apoptotic genes. The non-canonical (alternative) NF- κ B pathway is activated by receptors that are involved in lymphoid tissue organogenesis and lymphocyte development, such as Lymphotoxin β receptor(LT β R), which induces processing of p100 to p52 and the nuclear accumulation of p52–RELB dimers, results in mucosal addressin cellular adhesion molecule-1 (MAdCAM-1), chemokine (C-C motif) ligand 19 (CCL19), CCL20, CCL25, interleukin (IL)-4, IL-10 responses.

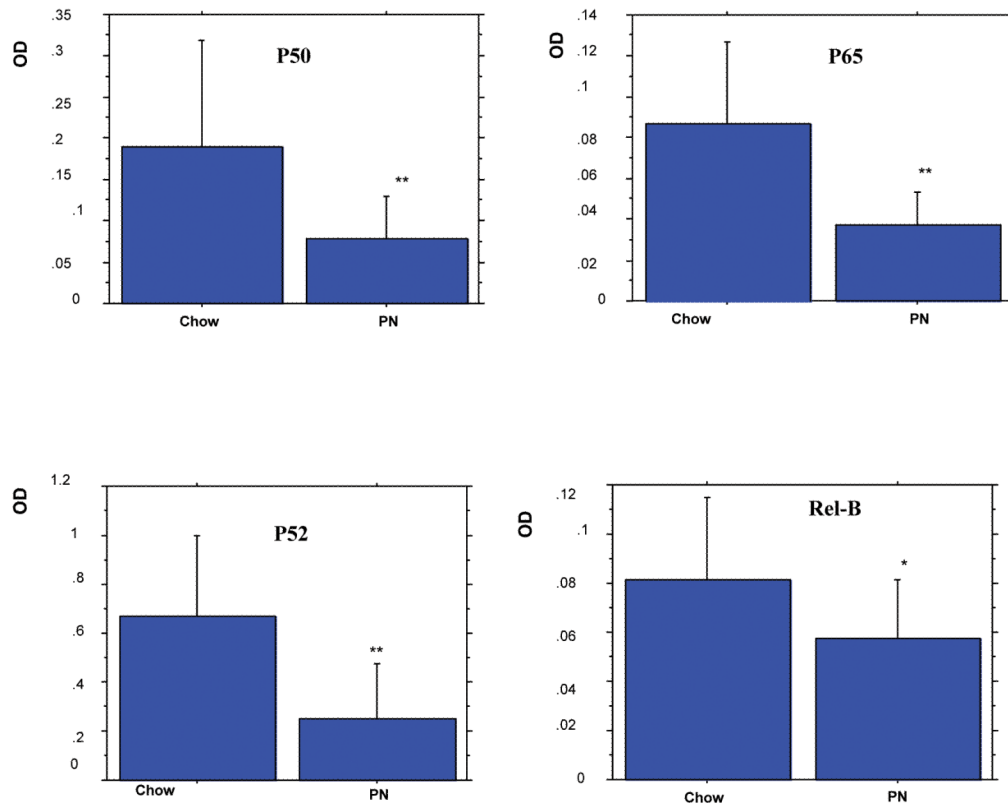


Figure 2.

Effects of parenteral nutrition (PN) on Nuclear Factor- κ B (NF- κ B) Activities in Peyer's patches (PP).

Data are expressed as mean OD \pm SD. Expt Chow (n= 19) vs PN (n=16). PN significantly reduced all Phosphorylated NF- κ B proteins in PP compared to Chow mice (* p<0.05, **p<0.01, PN vs Chow).

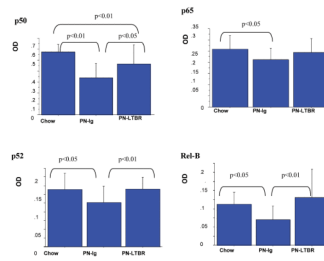


Figure 3.

Lymphotoxin β receptor (LT β R) Agonistic Antibody Stimulation of Nuclear Factor- κ B (NF- κ B) Activity in Peyer's patches (PP).

Data are expressed as mean OD \pm SD. Stimulation of PN mice with LT β R agonistic antibody significantly increased inflammatory product p50 as well as the non-canonical p52 and Rel B in PP compared with PN controls. There were no significant differences in p65 or the non-canonical levels of p52 and Rel B between PN-LT β R and Chow mice. LT β R stimulation of PN animals failed to improve p50 to Chow levels.

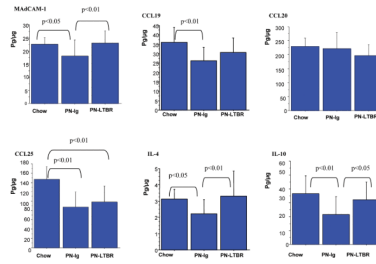


Figure 4.

Data were expressed as mean± SD pg/μg protein. PN significantly reduced MAdCAM-1, CCL19, CCL25, IL-4 and IL-10 compared to Chow mice. Compared to PN-Control mice, PN-LTβR significantly increased MAdCAM-1, IL-4 and IL-10 but not CCL20 and CCL25 in PP. There were no significant differences between PN-LTβR and Chow mice in levels of MAdCAM-1, CCL19, CCL20, IL-4 and IL-10.

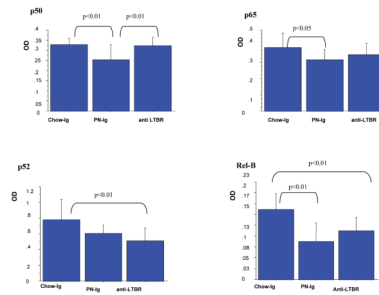


Figure 5.

Data are expressed as mean OD \pm SD. Blockade of LT β R in Chow mice with LT β R-Ig fusion protein significantly reduced the non-canonical products of p52 (0.51 ± 0.16 vs 0.78 ± 0.26 , $p < 0.01$) and Rel B (0.11 ± 0.03 vs 0.16 ± 0.04 , $p < 0.01$) to PN levels but had no effect on the inflammatory pathway products of p50 and p65 compared with Chow. As in experiment 1, PN reduced all NF- κ B pathway proteins.

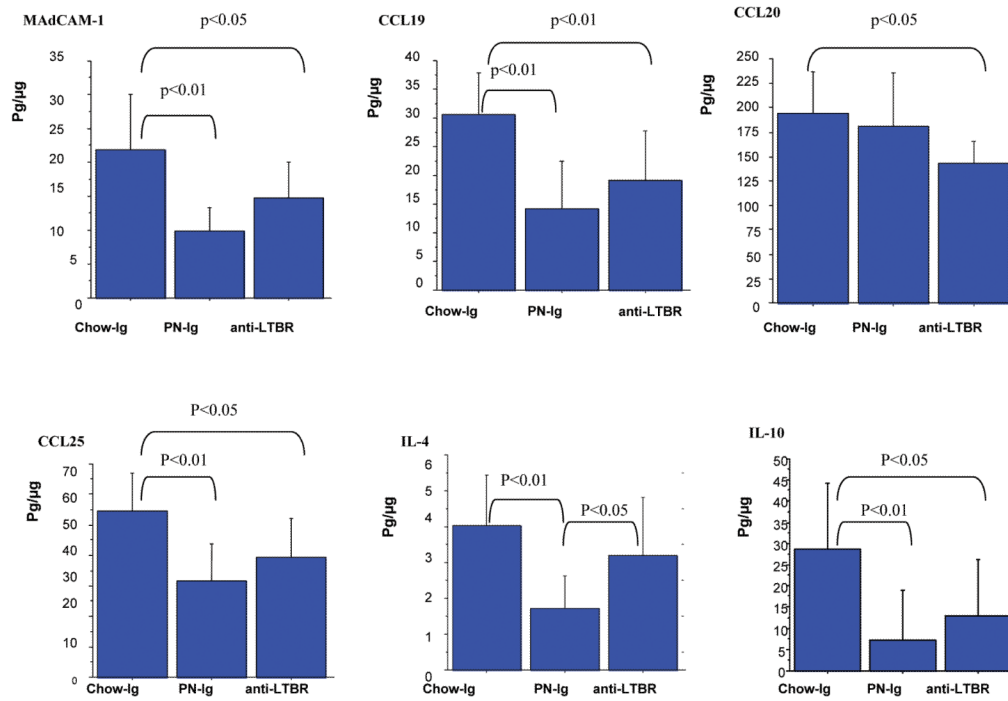


Figure 6.

Data were expressed as mean \pm SD pg/ μ g protein. The blockade of LT β R with LT β R-Ig fusion protein significantly reduced levels of MAdCAM-1, CCL19, CCL20, CCL25, IL-4 and IL-10, compared with Chow mice. There were no significant differences between Chow- anti LT β R and PN-Ig in levels of MAdCAM-1, CCL19, CCL20, CCL25 and IL-10, except for IL-4.