Parenteral and Elemental Enteral Nutrition Decreases Intestine Mucosal Immunity,

Which is Partially Restored by Dietary Proanthocyanidins and IL-25

By

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Dietary intake is disrupted in patients with contraindication to normal feeding. In these patients the use of enteral nutrition (EN), where nutrients are administered as a liquid directly into the gastrointestinal (GI) tract, and parenteral nutrition (PN), where nutrients are provided intravenously and bypass the GI tract completely, is utilized to prevent progressive malnutrition. Unfortunately PN feeding is associated with an increased risk of infection compared to enteral diets, especially in the critically ill. Previous work demonstrates PN decreases mucosal immune function and mucosal responsiveness to injurious or infectious challenge in the small intestine. Investigations of these alterations have largely focused on the adaptive arm of the mucosal immune system, defining changes in the Gut-Associated Lymphoid Tissue (GALT) that include decreased lamina propria and Peyer's patch lymphocyte numbers; decreased Th2 cytokines in the intestinal mucosa; lower mucosal levels of the secretory immunoglobulin-A (sIgA) transport protein, polymeric immunoglobulin receptor (pIgR); and decreased luminal levels of sIgA. Few investigations have focused on aspects of innate immunity, such as the barrier function of the intestinal mucosa, and no previous work has focused on the effect of PN or EN on Paneth cells or antimicrobial products. The core hypothesis of this dissertation is that mucosal barrier

function of the small intestine is reduced during decreased enteral intake and maintenance of the mucosal barrier with therapeutic approaches may protect the intestinal mucosa.

Intestinal barrier function is comprised, in part, of mucin glycoproteins secretion from goblet cells and antimicrobial compounds released from Paneth cells, including $sPLA_2$, Lysozyme, RegIIIγ, and defensins. These antimicrobial compounds, along with sIgA, localize to the mucous glycoprotein layer and provide a physical-chemical barrier to the underlying epithelial mucosa. Goblet and Paneth cells are regulated in-part by cytokine signaling from underlying lamina propria and intraepithelial cells. Mucosal cytokine changes can be measured directly, but pathways through which the cytokines signal can also be quantified, including the Janus Kinase/Signal Transducers and Activators of Transcription (JAK-STAT) pathway, one of the principle signaling mechanisms for immune cell proliferation and function.

In exploring the core hypothesis, the first focus of this dissertation is to investigate the effect of reduced enteral intake that occurs with PN and EN upon alterations in secretion and bactericidal activity of the Paneth cell protein $sPLA_2$ compared with Chow feeding (Chapters 2 and 3). The second focus of this dissertation is to examine the effect of adding physiological levels a class of flavonoids compounds isolated from cranberries, proanthocyanidins (PACs), to elemental EN to investigate potential protective effects on mucosal barrier function through changes in Paneth and goblet cell products, alterations in tissue cytokines and JAK/STAT signaling, and changes in sIgA secretion (Chapters 4, 5, and 6). The third focus on this dissertation is to examine the exogenous administration of a powerful Th2 cytokine, IL-25, to PN to investigate the stimulatory effects upon innate mucosal barrier function (Chapters 7 and 9). The final focus of this dissertation is to functionally assess mucosal barrier function of the small

intestinal through the development of an *ex vivo* intestinal segment culture (EVISC) model. The EVISC modeling allows for the investigation of PN induced vulnerability to *E. coli* enteroinvasion, changes in mucosal secretion of the antimicrobial compound sPLA$_2$, and whether these outcomes are modulated by the exogenous administration of IL-25 during PN (Chapter 8 and 9).

Dedication

This dissertation is dedicated to my parents, Nancy L. Esidor and Dr. Douglas F. Pierre, for always believing in me, never allowing negativity to discourage my dreams, and inspiring the determination in me to achieve my goals. I love you both. Thank you for bringing me into this world. I also want to dedicate this dissertation to my late grandfather, Dr. Joseph J. Pierre, who continues to be an inspiration in my life.

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Abbreviations Used

EN, Enteral Nutrition; PN, Parenteral Nutrition; GALT, Gut-Associated Lymphoid Tissue; IEL,
Intra-epithelial Lymphocytes; Th1, T Lymphocyte Helper 1; Th2, T Lymphocyte Helper 2; Treg,
Regulatory T Lymphocyte; PP, Peyer's patch; MadCAM-1, Mucosal Addresin Cellular
Adhesion Molecule 1; sIgA, Secretory Immunoglobulin A; pIgR, Polymeric Immunoglobulin
Receptor ; $sPLA_2$, Secretory Phospholipase A2 ; PAMP, Pathogen Associated Molecular Pattern;
mRNA, messenger Ribonucleic Acid; MDP, Muramyl Dipeptide; PRR, Pathogen Recognition
Receptor; NOD2, Nucleotide Oligomerization Domain 2; MUC2, Mucin 2; JAK/STAT, Janus
Kinase ; PACs, Proanthocyanidins; DP, Degree of Polymerization; γTCR, gamma T cell receptor;
EVISC, Ex Vivo Intestinal Segment Culture; (p)IVOC, (polarized) In Vitro Organ Culture; DES,
Decreased Enteral Stimulation

Chapter 1: Literature Review

Introduction

Decreased enteral intake occurs in patients with contraindication to normal enteral feeding. To provide nutrients to these patients, liquid nutrition is administered directly to the gastrointestinal tract by tube as enteral nutrition (EN) or provided intravenously as parenteral nutrition (PN) where it bypasses the gastrointestinal tract completely. This thesis is focused on the effects of decreased enteral intake that occurs with EN and PN upon mucosal barrier function in the small intestine. <u>My core hypothesis is that mucosal barrier function in the small intestine is decreased during reduced enteral intake and that maintenance of mucosal barrier function with therapeutics may provide protection to the intestinal mucosa.</u> In exploring my core hypothesis, the first focus of this dissertation is to examine the effect of reduced enteral intake that occurs with PN and EN to investigate alterations in secretion and bactericidal function of the Paneth cell protein $sPLA_2$ (Chapters 2 and 3). I hypothesize the administration of PN and EN will result in decreased Paneth cell secretion of $sPLA_2$ and reduced bactericidal function. The second focus of this dissertation is to investigate the effect of adding physiological levels of a cranberry derived class of flavonoids, proanthocyanidins (PACs), to elemental EN to investigate potential protective effects upon mucosal barrier function through changes in Paneth and goblet cell products, alterations in tissue cytokines and JAK/STAT signaling, and changes related to GALT mediated sIgA secretion (Chapters 4, 5, and 6). I hypothesize the addition of PACs to elemental EN will result in maintained barrier function compared with elemental EN alone. The third focus on this dissertation is to investigate the exogenous administration of a powerful Th2 cytokine, IL-25, during PN to explore potential stimulatory effects upon innate mucosal barrier function

(Chapters 7 and 9). Since Th2 cytokines are down regulated during long term PN feeding, I hypothesize the administration of IL-25 to PN will restore Paneth and goblet cell product secretions and result in increased mucosal barrier function in the small bowel. The final focus of this dissertation is to functionally assess mucosal barrier function of the small intestinal through the development of an *ex vivo* intestinal segment culture (EVISC). The EVISC modeling allows for the assessment of PN induced vulnerability to *E. coli* enteroinvasion, changes in mucosal secretion of the antimicrobial compound $sPLA_2$, and whether these effects are modulated by the exogenous administration of IL-25 during PN (Chapter 8 and 9). I hypothesize PN will lead to increased susceptibility to bacterial enteroinvasion associated with reduced secretion of $sPLA_2$ and the administration of IL-25 during PN will reverse these outcomes.

This literature review focuses on: 1) identifying the problem associated with decreased enteral intake; 2) describing the roles of the gut-associated lymphoid tissue (GALT) and specialized secreting epithelial cells in maintaining the mucosal barrier; 3) introducing proanthocyanidns (PACs) and IL-25 and the justifications for these therapeutics in maintaining intestinal barrier function during EN and PN, respectively; and finally, 4) describing an *ex vivo* model to functionally examine small bowel mucosal barrier function against an enteropathogenic bacteria following PN and the therapeutic effects of administering IL-25 during PN.

Identifying the Problem with Reduced Enteral Intake

PN has been used successfully as an alternative route of nutrition support since the 1960's. The implementation of PN marked an important advancement in the treatment of patients with contraindication to enteral feeding due to bowel trauma, gastrointestinal resection, or the

need for bowel rest due to inflammation that allowed nutritional care in patients otherwise destined to starve.[1] Both routes of alimentation, EN and PN, are clinical strategies (often in combination) to prevent malnutrition in patients who cannot eat normally and serve as lifesaving clinical strategies.

In the 1980's laboratory experiments investigating the effect of protein malnutrition and refeeding prior to intraperitoneal sepsis observed PN fed animals had the lowest survival rates.[2] Initially, it was hypothesized that deficiencies in the PN solution may have contributed to the outcome. This was tested by randomizing animals to PN formula administered either orally or intravenously and then challenging them with sepsis.[3, 4] The results demonstrated animals administered oral PN solution survived significantly better than those fed PN intravenously, ruling out an inherent problem with the PN formula and highlighting the initial link between the route of nutrition and maintenance of immune function. Since then, large clinical studies have confirmed these results and demonstrate PN is associated with an increased risk of intra-abdominal abscess and pneumonia in the critically ill compared with enteral feeding.[5] The observed susceptibilities are multivariable and likely include suppressed immune and mucosal barrier function in the small intestine, altered microbiome composition, heightened inflammatory responsiveness, and extremes in the gut lumen environment following the absence of dietary nutrients and the use of antibiotics. While many aspects of decreased adaptive immune function in regard to GALT have been described during PN, few investigations have focused on alterations to small bowel mucosal barrier function that may contribute to PN associated susceptibilities.

Overview of the Intestine as an Immune Organ

The intestine is a complex organ responsible for digestion, sensing of the environment, and development and maintenance of immune functions.[6] One important component of mucosal immune function is the intestinal epithelium, which acts as a physical barrier but also interactively samples the lumen and secretes molecules that protect the mucosa form microorganisms and foodstuff. The mucosal epithelium safe guards a surface area of around 200-300 m^2 (in humans) from food, environmental antigen and roughly 100 trillion resident bacteria that increase in number moving distally through the gastrointestinal tract.[7, 8] Despite this immense challenge, the epithelium consists of a single layer of continuous columnar epithelial cells held together by tight junction proteins. While the mucosa faces large loads of potentially harmful antigen passing through the gastrointestinal tract, few pathogens succeed in breaching the epithelial defenses and enter systemic circulation in healthy individuals, a credit to the effectiveness of this extremely robust system.[9]

Beneath the epithelium a large mass of cells types reside in the lamina propria (LP) and intraepithelial (IEL) spaces. Collectively, the lymphoid cells that support the intestinal mucosa comprise the GALT, in which nearly 70% of immunologically active immune cells in the body are located.[10] The chief GALT function is the sampling of intestinal antigen and the subsequent release of antigen-specific and non-specific secretory Immunoglobulin A (sIgA) upon the intestinal mucosa.[10] There are two primary effecter lymphoid subsets, the T helper 1 (Th1) and T helper 2 (Th2) lymphocytes. Reponses driven by the adaptive immune arm, such as release of sIgA, are mediated by Th2 cytokines and are largely referred to as anti-inflammatory profiles.[11] The classical innate responses, such as the recognition of bacterial and viral components that

breach the mucosa, are mediated Th1 cytokines and are referred to as pro-inflammatory cytokine profiles.[12] Additionally, at least two regulatory T cell subsets assist in coordinating the effector T lymphocyte subsets. These include the T regulatory (Treg) lymphocytes and T inflammatory (Ti) or T17 lymphocytes.[13, 14] Under normal conditions these subsets work together to orchestrate immune function in the gut. The lymphocyte subset classifications are not absolute, since a considerable degree of cooperation and overlap exists between various subsets, however, these conventions allow us to classify and describe immune responses to improve our understanding of the complex interactions.

The Role of GALT and IgA at the Mucosal Barrier

The steps beginning with PP sampling of luminal antigen that result in sIgA release at mucosal surfaces are collectively known as the common mucosal immune hypothesis [Figure 1].[15, 16] The release of sIgA is the most important function of GALT, since sIgA is the primary defense molecule against pathogens at mucosal surfaces.[17]

The GALT is compartmentalized into induction sites, maturation sites, and effector sites. Peyer's Patches (PP) and lymphoid follicles serve as induction sites in humans and mice, where intestinal lumen sample is taken up through phagocytosis by specialized epithelial cells called microfold (M) cells.[16] Tonsils are arguably also induction sites that belong to the GALT. Antigen is gathered by dendritic cells in the sub-epithelial dome of PP or lymphoid follicles that present it to specialized subsets of T and B lymphocytes that display the surface markers α4β7 and L-selectin. These surface markers interact with a modified form of mucosal adressin cellular adhesion molecule 1 (MadCAM-1) on high endothelial venules that direct the lymphocytes to the

intestinal induction sites.[18, 19] Once sensitized to antigen, the α4β7 lymphocytes migrate to the

mesenteric lymph nodes where they undergo maturation and proliferation, which primes these

cells towards an activated state.[16] The maturation process includes up-regulation of the α4β7

surface marker and suppression of L-selectin for future interaction with the unmodified form of

MadCAM-1 at mucosal effector sites.[20] The lymphocytes then leave the mesenteric lymph nodes

and exit the lymphatic system through the thoracic duct, enter circulation, and home to mucosal

surfaces, including the intestinal, respiratory, mammary, nasal passage, and urogenital tracts.[21]
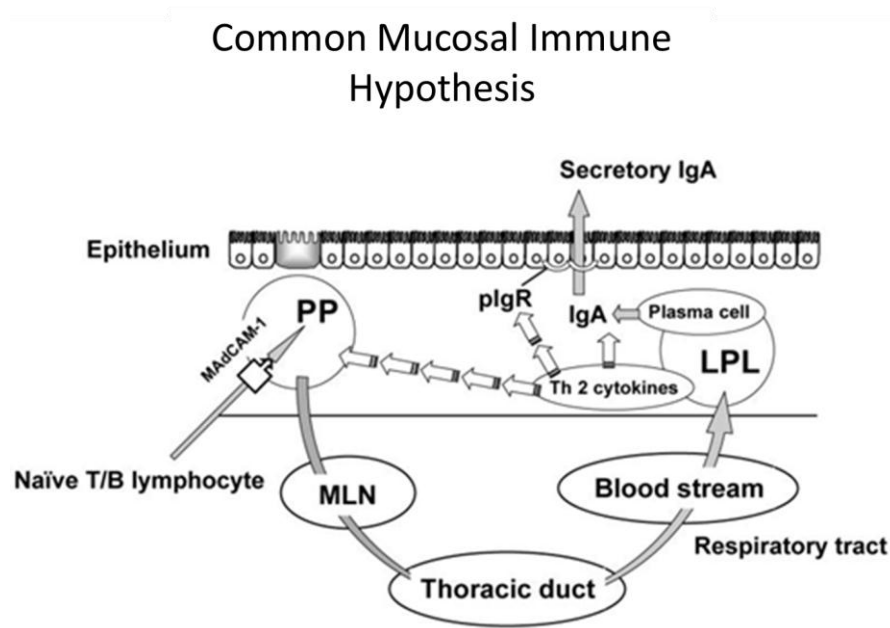
Figure 1. *Schematic of the common mucosal immune hypothesis. MAdCAM-1: mucosal addressin cellular adhesion moleucle -1; PP: peyer's patch; MLN: mesenteric lymph node; LPL: lamina propria lymphocytes; pIgR: polymeric immunoglobulin receptor. Adapted from.[1]*

In the intestine, the effector site is the lamina propria and does not contain organized architecture. Instead, cells are loosely arranged beneath the epithelium layer within the intestinal villus and submucosa. Within the lamina propria mature B lymphocytes, called plasma cells, produce and release sIgA.[16] sIgA is a dimeric form of immunoglobulin that is complexed by J chain, a peptide which protects the dimeric conjugation from cleavage by digestive and bacterial enzymes. The dimeric sIgA is received at the basolateral surface of enterocytes by the transport protein polymeric immunoglobulin receptor (pIgR), transcytosed across the enterocyte, and released at the mucosal surface into the lumen.[22] The production and release of sIgA by plasma cells and the transport of sIgA by enterocyte pIgR is supported by the cytokines Th2 cytokines IL-4, IL-10, and IL-13.[23] These cytokines are produced by a number of immune cells, and perhaps epithelial cells, along with other Th2 cytokines, IL-5, IL-9, IL-10, and IL-25.[11]

A reduction in nutrient intake disrupts the efficiency and structure of GALT leading to reduced levels of sIgA at mucosal surfaces.[24] First, a reduction in splanchnic blood flow follows reduced enteral feeding carrying fewer α4β7 lymphocytes for interaction with the Peyers patch and lymphoid follicle MadCAM-1.[21] Additionally, NF-kB, a nuclear factor responsible for MadCAM-1 expression, is suppressed with decreased enteral stimulation.[25] As fewer cells enter the Peyer's Patch, due to reduced circulating numbers and suppressed MadCAM-1 expression, fewer lymphocytes are sensitized to antigen. This precedes a reduction in lymphocyte maturation in mesenteric lymph nodes and subsequently fewer lymphocytes populating the lamina propria of the intestine and other mucosal surface effector sites. As a consequence, reduced levels of Th2 cytokines are produced by the depleted T lymphocyte numbers and less sIgA is produced by reduced plasma cell counts.[26] sIgA binds bacteria, inducing opsonization and neutralization of

bacterial virulence. Furthermore, when bacteria that are opsonized by sIgA are sampled by PP they stimulate less inflammatory responses than non-opsonized bacteria. Therefore, the loss of sIgA leads to an increased risk of bacterial adherence to the host and greater mucosal inflammatory responses.[27]

The Role of Specialized Secreting Epithelial Cells of the Mucosal Barrier

While organization and regulation of the adaptive immune functions that take place within the GALT is recognized, a less appreciated aspect of immune function is the specialized secreting epithelial cells that help comprise the epithelial layer [Figure 2]. Maintaining the barrier between the host and the luminal environment is performed at the most basic level through the formation of a physical barrier.[6] The intestinal lumen is lined by a continuous monolayer of epithelial cells, including absorptive enterocytes, hormone secreting enteroendocrine cells, mucin producing goblet cells, antimicrobial producing Paneth cells, and M cells that reside on surfaces of PP and lymphoid follicles.[9] Each of these epithelial cell types are derived from the pluripotent stem cells at the base of the intestinal crypts of Lieberkuhn. In addition to forming a physical barrier, Paneth and goblet cells, and to a lesser extent enterocytes, also secrete physico-chemical compounds which are vital in protecting the mucosa from insult and interact with luminal microbiota.[28, 29] In investigating the physico-chemical barrier, this dissertation largely focuses on Paneth and goblet cells.

Paneth cells remain at the base of the crypt of lieberkuhn after differentiation and live 20-30 days.[28] Paneth cells produce and secrete an array of antimicrobial peptides and proteins, including lysozyme, secretory Phospholipase A_2 group IIA ($sPLA_2$), RegIIIγ, and defensins or

cryptidins.[30] Antimicrobial products from Paneth cells to modulate microflora populations and the loss of these compounds is associated with mucosal inflammation and colitis. Paneth cells exocytose their granular and cytosolic products into the gut lumen in response to cholinergic stimulus and the presence of bacterial compounds.[31] Of these antimicrobial products, $sPLA_2$ functions catalytically to cleave fatty acids from glycerol backbones from phospholipids in cellular membranes.[32] With an overall cationic charge, $sPLA_2$ is drawn to negatively charged bacterial cell membranes within the gut lumen where it functions to induce membrane permeability and lysis.[33] $sPLA_2$ has been shown to induce bacterial lysis of gram-positive and gram-negative bacterial strains in vitro and appears to preferentially attack membranes sites involved in growth.[34] Lysozyme is concentrated in the cytosol and functions to help detect bacteria taken up by Paneth cells from the crypt. Lysozyme cleaves the pathogen associated molecular pattern (PAMP) muramyl dipeptide (MDP) from peptidoglycan residues found in bacterial cell walls. This dipeptide is the ligand for the pathogen recognition receptor (PRR) intracellular nucleotide- oligermerization domain 2 (NOD2) and this interaction stimulates production and secretion of Paneth cell products. This sensing mechanism is conserved across a broad range of species, highlighting the importance of these receptors in host defense. Furthermore, the importance of the NOD2 receptor is recognized in Crohn's disease, where 30% of cases are associated by mutations in the NOD2 transcription sequence.[35] Defensins in humans and cryptidins in rodents are pore forming peptides that attack bacterial membranes and induce lysis through membrane permeability. Together, these compounds provide the host with the potential to modulate bacterial growth and composition with the gut lumen.[31]

Surprisingly, no previous work has focused on the effect of PN or EN on Paneth cells or

its products. One study examined the effect of starvation upon Paneth cells, which demonstrated

48 hours of starvation leads to decreased Paneth cell lysozyme, RegIIIγ, and cryptidin mRNA,

suppressed lysozyme and RegIIIγ protein, and increased bacterial translocation across the

mucosa.[36] Therefore, the first focus of this dissertation is investigating the effect of decreased

enteral intake to investigate secretion of the Paneth cell protein sPLA$_2$ (Chapters 2 and 3).
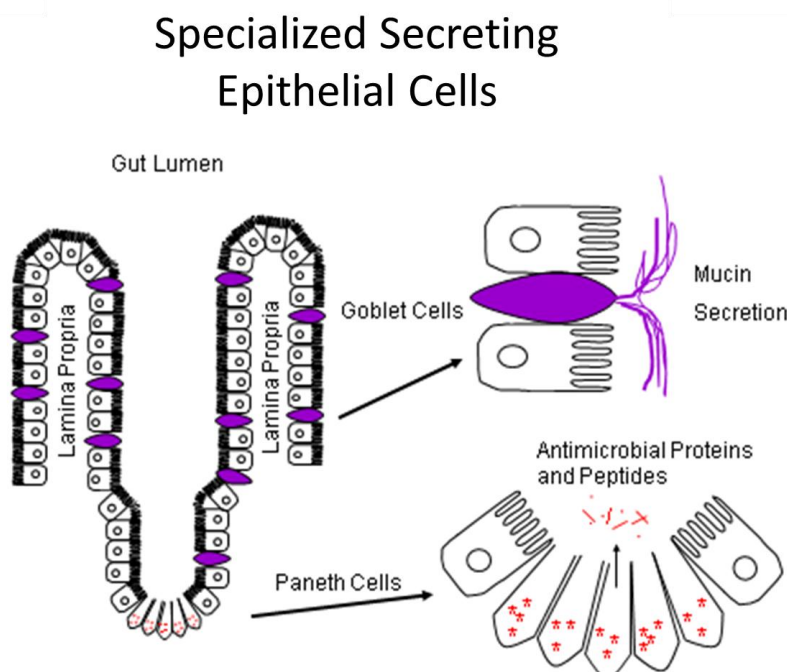
Figure 2. *Specialized secreting epithelial cells of the mucosal barrier.*

Goblet cells are specialized mucin glycoprotein secreting cells which, unlike Paneth cells,

migrate up the villi after differentiation and turn over with the epithelial layer every 3-5 days.[37]

The major mucin glycoprotein secreted in the intestine of both mice and man is the highly

oligomeric mucin2 (MUC2) and loss of its expression in mice (MUC2-/-) leads to lethal colitis

by early adulthood.[38] Therefore, the contribution of MUC2 to the mucus layer appears essential in maintaining barrier function and a healthy mucosa.[38] A large number of other mucins are found throughout mucosal surfaces in various ratios as secreted and membrane-bound forms. Goblet cells also produce trefoil peptides, which promote epithelial growth and repair.[37] While regulation of goblet cell products is not completely understood, release of mucins has been demonstrated following cholinergic stimulation, similar to Paneth cells.[39]

The secreted Paneth cell antimicrobial products, $sPLA_2$, Lysozyme P, and the defensins, along with the adaptive immune molecule sIgA, localize in high concentrations to the mucin glycoprotein layer secreted by goblet cells.[40] One underlying mechanism for this localization is charge induced interactions between the cationic Paneth cell antimicrobials and anionic mucins glycoproteins. Additionally, the sIgA J chain appears to have high affinity to intestinal mucins. Together, the mucus layer loosely overlies the surface of the small intestinal epithelium, but firmly over the large intestinal epithelium.[29] Functionally, this layer provides a viscous surface for digesta to pass over the epithelium during peristalsis, but is important in preserving the integrity of the mucosa through prevention of mucosal access to pathogens. Further, the composition of antimicrobial compounds within the mucin layer may influence the microbiota, since specific microorganisms appear to colonize and interact with this layer when the axial structure of the microbiome is examined.[41]

Studies examining changes to intestinal goblet cells following PN have been performed in neonatal piglets and adult rats. In neonatal piglets PN induces apoptosis of mucosal epithelial cells but selective survival of goblet cells leading to a relative increase in goblet cell numbers.[42] It is important to note many neonatal mammals, including humans, do not initiate adaptive

immune function until early adolescence and up-regulation of barrier function may be a basic

compensatory mechanism in the absence of sIgA. Studies in adult rats have inconsistent

outcomes, sometimes observing increases in goblet cell numbers while others report no change.[43,] [44] This may be due to variability in sample site regionalization, PN formulation, and study length.

Consistent, however, is an observed reduction in the luminal glycoprotein MUC2 in both animal

models, an effect also observed in humans following PN. While the mucosal remodeling in

regard to Goblet cells varies between animal models, goblet cell function appears to decrease as

measured through reduced secretion of mucin glycoproteins.

In contrast, experiments examining EN or elemental EN effects on goblet cells and

intestinal remodeling consistently demonstrate reduced goblet cell numbers and size in the distal

ileum.[45] The discrepancy between PN and EN effects on goblet cells may be a function of the

altered amino acid requirement. Threonine accounts for greater than 30% of intestinal

glycoprotein amino acid residues and its availability may alter glycoprotein production.[46] Dietary

route influences amino acid availability. First, depending upon administration rates elemental EN

can be completely absorbed in the first $1/3^{rd}$ of the small intestine. Once taken up by enterocytes,

many amino acids are metabolized by the intestinal tissue before entering the portal vein for

further clearance by the liver. Therefore, the distal intestinal mucosa receives nutrients only from

circulation on the basolateral surface during elemental EN. Consequentially, since the intestine

can metabolize large amounts of ingested amino acids, the estimated threonine requirement for

PN is only 45% of the oral requirement.[47] Furthermore, while the mucosal turnover rate is

reduced with PN and EN, enteral nutrients in the proximal intestine may stimulate GI hormones

that may influence mucosal remodeling.[48] Thus, the administration of PN solution compared

with EN solution that is isocaloric, isonitrogenous, and contains similar amino acid compositions, may result in relative excess of threonine administration in PN. Based on these considerations, differences may be expected in mucosal remodeling between PN and EN and may be important in understanding barrier function during reduced enteral intake.

The Regulation of Specialized Secreting Epithelial Cells of the Mucosal Barrier

The mucosal barrier is influenced by the underlying GALT lamina propria and intraepithelial cells and signaling from the enteric nervous system. The epithelium is regulated by chemokines, cytokines, and neuropeptides.[16, 48, 49] Recent observations also speculate the presence of luminal cytokines may play important paracrine roles in epithelial signaling. Several examples illustrate a link between the epithelium and the GALT compartment. For example, an anti-rejection drug given to organ transplant patients, alemtuzumab, reduces lamina propria T lymphocyte numbers and decreases expression of Paneth cell products.[50] The disruption of Paneth cells with alemtuzumab drastically disrupts the gut microbiome. In contrast, stimulating lamina propria T lymphocytes is associated with increased Paneth cell function.[51] Examples also exist for interactions between GALT lymphocytes and goblet cells, where inhibiting the αβ or γδ T cell receptors depletes goblet cell numbers in the mucosa.[52]

While the overall understanding of Paneth and goblet cell regulation is not complete, experiments demonstrate GALT cytokines that function through the Janus Kinase/ Signal Transducers and Activators of Transcription (JAK/STAT) pathway play an important role. JAK/STAT is one pleiotropic signaling cascade used that serves as a primary signaling mechanism for a wide array of cytokines,[53] including cytokines that regulate immune cell

development and function. Therefore, this cellular pathway contains numerous markers that can be measured to quantify cytokine signaling. Goblet and Paneth cells are non-hematopoietic derived cell types on which the Th2 cytokines IL-4 and IL-13 bind IL-4R, comprised of an IL-4 receptor-α and IL-13 receptor- α1 component.[54] Binding of IL-4 to the IL-4 receptor-α component leads to JAK1 phosphorylation. Binding of IL-13 to the IL-13 receptor-α1 leads to phosphorylation of JAK2 and TYK2.[53] Phosphorylation of these intracellular proteins stimulates phosphorylation of the nuclear transcription factor STAT6, otherwise latent in the cytosolic compartment. Once phosphorylated STAT6 (PSTAT6) stimulates transcription of several important products involved in barrier function. Specifically, increased levels of PSTAT6 in Paneth cells induces transcription of $sPLA_2$ and Angiogenin4.[55] In goblet cells, increased levels of PSTAT6 induces cellular hyperplasia and increased expression of MUC2 and Trefoil factor, a molecule that stimulates epithelial repair.[52, 56] Other inducers of the JAK/STAT pathway also appear involved in epithelial regulation including MAP kinase, which has been shown to independently stimulate goblet cells.[53]

The Role of Dietary Proanthocyanidins upon Mucosal Barrier Function

Flavonoids are a subclass of polyphenols that contain the structure of two phenolic rings linked by a 3 carbon chain [Figure 3].[57] Within this basic structure, structural heterogeneity exists depending on oxidation state, polymerization, and other modifications. Flavonoids are thought to protect plants from a wide range of insults, including UV radiation, and insect and animal consumption. Among the various flavonoid subclasses perhaps the most unique are

proanthocyanidins (PACs), or condensed tannins, which are oligomers of flavan-3-ol subunits [Figure 4].[58]
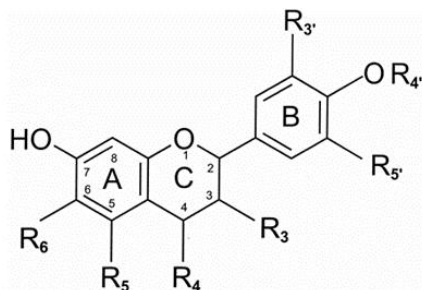
Figure 3. *Basic flavonoid structure with each ring and carbon labeled.*
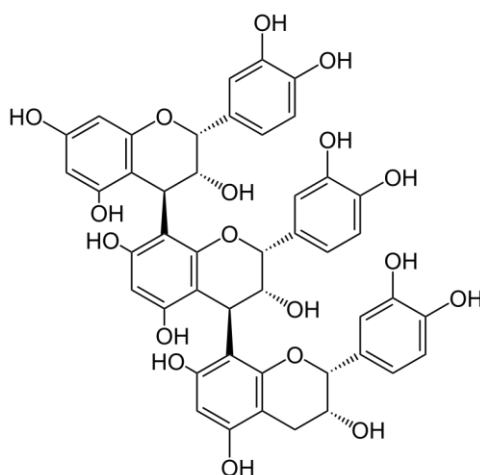
Figure 4. *Proanthocyanidin trimer structure, with three flavan-3-ol units linked by 4→8 carbon bonds.*

PACs are widely distributed in primate and human dietary sources, including fruits, grapes, cranberries, and apples, and other foods and beverages such as chocolate and wine.[59] Epidemiological studies suggest PACs are associated with a range of beneficial health effects, including the prevention of some chronic diseases.[60] However, PACs are minimally transported

across the enterocyte layer due to non-hydrolysable bonds between the flavan-3-ol subunits and their propensity to complex both dietary and endogenous proteins.[61] Further, PACs are unique among polyphenol compounds in that their oligomeric structures range in degrees of polymerization (DP) between 3 to 26, or more. Therefore, PACs have higher molecular weights than other common flavonoids and rodent studies suggest greater than 95% of PACs remain in the intestinal lumen during transit through the gastrointestinal tract. A recent human study concluded that such miniscule levels of PACs were detectable systemically that there is reason to doubt on a direct role for these compounds outside of the gut.[62]

Since PACs are poorly absorbed, mechanisms of action within the GI tract have been investigated to explain their potential beneficial effects. PACs are demonstrated to exert antioxidant, anti-inflammatory, and non-specific antimicrobial functions within the gut.[60] Antioxidant capacity is frequently measured by reduction in reactive oxygen species. Anti-inflammatory effects have been observed through decreased NF-kβ expression, lower pro-inflammatory tissue cytokines, and reduced STAT1 phosphoyrlation.[60] Animal studies also demonstrate the addition of dietary PACs palliates chemically-induced colitis and gastric ulceration and are associated with greater tissue levels of the Th2 cytokine IL-4.[63, 64] The current body of literature lacks investigations that elucidate the role of PACs upon the GALT and mucosal barrier.

PACs form complexes with proteins through hydrogen bonding and hydrophobic interactions. Understanding these mechanisms is important for understanding the role of PACs in the GI tract. In the oral cavity, crosslinking occurs between PACs and salivary glycoproteins inducing precipitation, a process that causes the characteristic astringency that many fruits and

beverages have that contain high levels of condensed tannins.[65] PACs with higher DP have greater potential for crosslinking and precipitation.[66] Several biological effects occur in response to PAC astringency that have been investigated in rodents, including increased salivary glycoprotein excretion, hypertrophy of the parotid gland, and shift in salivary composition to proline rich proteins that resist complexation with PAC.[67] Considering these effects, it is possible that the response to PACs complexation may continue throughout the GI tract and that PACs may have underappreciated interactions with GALT induction sites, specifically PPs and lymphoid follicles.

In tangent with stimulation of PP, a subset of intestinal T lymphocytes are situated within the epithium, the IEL T cells. *In vitro* studies demonstrate γδ IEL T lymphocytes respond to PACs through activation and proliferation.[68] Interestingly, the level of γδ T cell response also increases with greater DP of PACs. Importantly, γδ T lymphocytes secrete greater levels of IL-4 and IL-13 per cell than αβ T lymphocytes at baseline and, as noted, the loss of the γ T cell receptor (γTCR) results in the loss of Goblet cells.[52] Since γδ T lymphocytes are a major population of IEL lymphocytes, being adjacent to the lumen, it is possible that PACs also influence these cells directly at the mucosal surface.

Therefore, the second focus on this dissertation is to investigate the effect of adding cranberry PACs to elemental EN to investigate the effects upon tissue cytokines, JAK/STAT regulation, alterations in Paneth and goblet cell products, and sIgA secretion, which may indicate of the effect of PACs upon mucosal barrier function (Chapters 4, 5, and 6).

The Role of Cytokine Immunomodulation upon Mucosal Barrier Function

In patients requiring long-term PN alone where the administration of enteral diet are not possible a variety of systemic stimulatory agents have been experimentally investigated to stimulate immune responsiveness and improve outcome parameters. These include increased levels of certain amino acids that may be limiting and beneficial when added in high concentrations, such as glutamine and arginine (see Appendix II), and direct immunomodulatory molecules such as neuropeptides and cytokines.[69-73] Cytokines offer a unique potential since they, unlike general stimulants, offer control of specific immune arms. This prospect is not without significant challenges considering the fine balancing between the Th1 and Th2 profiles, and other cytokine profiles, that normally have tight regulatory control over immune function.

Short periods of reduced enteral intake are followed by a reduction in gut function while the bowel is at rest to conserve energy. These responses are appropriate for short term cessations in feeding and digestion. During extended periods of reduced enteral intake, such as occur with long term PN, Th2 cytokines are suppressed disproportionally compared with the elevated Th1 cytokines driven by luminal environment changes, such as decreased pH, increased bacterial virulence, and the lack of nutrients.[1, 74] Th1 cytokines such as IL-1β and TNF-a, suppress Th2 cytokine profiles, promote inflammation, and increase pro-inflammatory eicosanoid production.[13] Th1 cytokines can also promote cellular stress and tissue damage when un-proportionally elevated. On the other hand, Th2 cytokines, such as IL-4, IL-9, and IL-13, suppress Th1 responses and have the ability to shift the intestinal mucosa from an absorptive state towards a secretion state.[11]

Since nutrient absorption is less important than maintaining mucosal integrity during long term PN, shifting the epithelium towards a secretion state with the use of Th2 cytokines may

prove an advantageous in protecting against hyper-inflammation and bowel pathogens. While many Th2 cytokines are known, including IL-4, IL-5, IL-9, IL-13, and IL-25, the use of IL-25 has recently been shown to strongly enhance each of the former Th2 cytokines in the gut.[75] The current understanding of IL-25 and its effects have largely come through studies of nematode-host interactions. Following infection with the parasites *Nippostrongylus brasilienusis, Trichinella spiralis*, or *Heligomosomoides polygyrus,* a large IL-25 response that appears to be driven by the epithelium stimulates smooth muscle, goblet cell, and Paneth cell hypertrophy.[76] Although these studies investigate IL-25 under a pathological state, its utilization is obvious in conditions where Th2 cytokines are deficient. IL-25 mRNA is found in epithelial cells, but not LP or IEL cells, suggesting the importance of this cytokine in barrier function and epithelial signaling.[77] Therefore, since decreased enteral stimulation induces a loss of specialized secreting epithelial cell function that precedes an increased risk of infection, the use of Th2 cytokines during PN, such as IL-25, may stimulate specialized epithelial secreting cells and decrease susceptibility to bacterial enteroinvasion. Thus, the third focus on this dissertation is to investigate the addition of IL-25 to PN to investigate stimulatory effects on the innate mucosal barrier, specifically changes to Paneth and goblet cell secretions and luminal levels of sIgA (Chapters 7 and 9). [5, 78]

The Modeling of Bacterial Enteroinvasion to Evaluate Mucosal Barrier Function

Abscess causing organisms include both aerobes, such as *Escherichia coli*, *Klebsiella, Streptococcus*, and *Enterobacter* species, as well as anaerobes, such as *Bacteroides,*

Peptostreptococcus, and *Clostridium* species.[45, 79] Currently, much of the work done to investigate the interaction of bacteria with the host is performed in cell monolayers; however, these models do not allow insight to the complex *in vivo* interactions that occur at the host-pathogen interface. A better understanding of these processes, especially through replication of a vulnerable host mucosal barrier, may allow for the development of more effective prophylaxis treatments and insights into pathogenic infection.

Models that study the intestinal mucosa directly have been reported, specifically the described methodologies of the conventional and polarized *In Vitro* Organ Culture (IVOC and pIVOC) that investigated attaching and effacing bacteria in human biopsies.[80-82] Others have utilized Ussing chambers to investigate bacterial translocation. Based on the pIVOC model, we optimized an *Ex Vivo* Intestinal Segment Culture (EVISC) model to quantity bacterial enteroinvasion of the mucosa, one of the primary steps of colonization and systemic bacterial translocation. The model also allows investigation of intestinal responsiveness to bacteria through detectable secretion of the Paneth cell antimicrobial products. As a final focus of this dissertation, the EVISC method was developed to investigate PN induced vulnerability to *E. coli* enteroinvasion, the mucosal secretion of the antimicrobial compound, $sPLA_2$, in response to enteroinvasion, and whether these parameters were modulated with exogenous administration of IL-25 during PN feeding (Chapter 8 and 9).

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Chapter 2

Route and Type of Nutrition and Surgical Stress Influence Secretory

Phospholipase A$_2$ Secretion of the Murine Small Intestine

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Abstract

Background: The function of sPLA$_2$ is site dependent. In tissue, sPLA$_2$ regulates eicosanoid production; in circulation, sPLA$_2$ primes neutrophils; and in the intestinal lumen, sPLA$_2$ provides innate bactericidal immunity as a defensin-related protein. Since parenteral nutrition (PN) primes leukocytes while suppressing intra-luminal mucosal immunity, we hypothesized 1) PN would diminish luminal sPLA$_2$ activity, but increase activity in intestinal tissue and serum and 2) stress would accentuate these changes.

Methods: Mice received Chow, Complex Enteral Diet (CED), intragastric PN (IG-PN), or PN in Experiment 1, and Chow, Chow+Stress, PN, and PN+Stress in Experiment 2.

Results: Expt1: Luminal sPLA$_2$ activity was greatest in Chow and decreased in CED (p=0.0001), IG-PN (p=0.0002), and PN (p=0.0001) with PN lower than CED (p<0.002) or IG-PN (p<0.0001). Compared to Chow, serum sPLA$_2$ activity dropped after CED (p = 0.042), IG-PN (p<0.0001), and PN (p=0.0004). Serum sPLA$_2$ was higher in portal than systemic serum (p=0.04).

Expt2: PN lowered luminal sPLA$_2$ activity vs Chow (p<0.0001). Stress lowered luminal sPLA$_2$ activity in Chow (p<0.0001), without change in PN. Following stress, luminal IgA increased in Chow (p=0.0025) but not PN (p=0.18). Serum sPLA$_2$ activity increased in PN (p<0.03).

Conclusions: Parenteral nutrition attenuates sPLA$_2$ activity in intestinal fluid consistent with suppressed innate mucosal defense. Stress suppresses luminal fluid sPLA$_2$ activity in Chow, but not the IgA response: PN impairs both. Stress significantly elevates serum sPLA$_2$ in PN fed mice consistent with known increased neutrophil priming with PN. PN reduces innate bactericidal immunity of the gut but up-regulates serum pro-inflammatory products post-stress.

CLINICAL RELEVANCY

The dynamics of the bacterial – host relationship results in a symbiotic which is beneficial to both. These relationships deteriorate in illness and following injury as both bacteria and the host adapt to changing environments in attempts to survive. Most of the recent research into these changes have focused on bacterial responses and changes in mucosal immunity (as the functional arm of adaptive immunity). The finding that parenteral feeding negatively affects gut bactericidal molecules after injury implicates impairment of the most basic level of the gut immune system - innate immunity – and failure to maintain the integrity of this basic anti-bacterial defense.

INTRODUCTION

Parenteral Nutrition (PN) prevents progressive malnutrition in patients with contraindications to enteral feeding. However, PN is associated with increased infectious complications and inflammation compared to enterally fed patients.[1-3] These risks may be partially attributable to increased host tissue inflammation and compromised mucosal immune mechanisms when the gut is not stimulated with enteral feeding.

Our laboratory previously demonstrated that route and type of nutrition affect numerous components of mucosal immunity particularly in the gut associated lymphoid tissue (GALT). As enteral stimulation increases from none (PN) to enteral administration of an elemental diet (intragastric PN:IG-PN) to enteral feeding of a complex enteral diet (CED) containing proteins, carbohydrates and fat to *ad libitum* feeding with chow, mucosal immune function increases with

higher levels of intestinal and respiratory IgA; greater lymphocyte counts in Peyer's patches, the intestinal lamina propria and lung tissue; increasing levels of IgA-stimulating cytokines in the gut; and an ability to respond to injury with increases in gut and airway IgA levels. Systemically, PN increases expression of ICAM-1 and p-selectin in the gut vasculature recruiting polymorphonuclear leukocytes (PMNs) while priming them for a subsequent insult.[4,5] Subsequent injury then augments PMN activation and increases tissue damage compared to animal fed enterally.[6] Within the GALT itself, PN impairs production and transport of Immunoglobulin-A (IgA),[7-10] which functions as an antibacterial and anti-inflammatory molecule. Furthermore, PN also eliminates the ability of the gut mucosa to increase IgA levels in response to injury. While IgA represents an adaptive immune molecule at mucosal surfaces, Paneth cells within gut mucosa also produce innate immune molecules, the defensins and other bactericidal proteins. Secretory phospholipase A_2 (sPLA$_2$) is involved in both processes: it functions as a defensin-related protein when released into the gut lumen but activates PMNs when elevated systemically.

sPLA$_2$ is a superfamily of lipolytic enzymes responsible for diverse regulatory functions in mammals and all sPLA$_2$ isoforms depend on millimolar concentrations of calcium for catalytic activity. The catalytic function of sPLA$_2$ hydrolyzes phospholipids from the sn-2 position of the glycerol backbone to release long-chain fatty acids from either bacterial or mammalian cell membranes, but with different outcomes. Paneth cells within the small intestine (SI) produce and secrete defensins, lysozyme P, and the sPLA$_2$-IIA isoform to provide bactericidal activity.[11] The cationic sPLA$_2$ enzyme attacks the negatively charged bacterial cell membranes inducing membrane permeability and lysis.[12-13] *In vitro,* sPLA$_2$-IIA kills gram-positive and several gram-

negative bacterial species[14-17] and may preferentially attack membrane sites involved in cellular growth.[18] $sPLA_2$-IIA over-expression in transgenic mice reduced the risk of septic mortality during bacterial challenge compared to PLA_2-deficient littermates.[19]

On the other hand, $sPLA_2$ within host tissue and the vascular system provides a pro-inflammatory function. The outer envelopes of mammalian cellular membranes are largely comprised of uncharged phosphotidylcholine and cholesterol. With its net cationic charge, $sPLA_2$ acts weakly on host tissue under normal circumstances. However, inflammatory conditions such as rheumatoid arthritis, [20, 21] acute pancreatitis,[22] and critical illness,[23,24] are characterized by rapid elevation in serum $sPLA_2$. Under these conditions elevated $sPLA_2$ correlates with increases in other serum and tissue inflammatory markers, such as TNF-α, IL-1, and IL-6.[25-27] Increased $sPLA_2$ releases free fatty acids which are metabolized to eicosanoids, e.g. prostaglandins, thromboxanes, and prostacyclins, which are powerful inflammatory mediators.[14] Additionally, $sPLA_2$ activates cell surface receptors on immune cells, such as PMNs, to prime them for augmented inflammatory responses. [28, 29]

PN exerts detrimental effects on the adaptive mucosal immune system of the gut and gut inflammatory processes but little is known about its effect on innate immunity. Since $sPLA_2$ is involved in both innate bactericidal activities of the gut lumen and host inflammation systemically through mediators and cell priming, we investigated alterations in $sPLA_2$ activity within the small intestine tissue, lumen, and serum after PN. We compared and contrasted these results with the responses of the adaptive mucosal immune system using our established model of surgical stress. We hypothesized that PN and surgical injury would suppress the defensin-related protein $sPLA_2$ in the intestinal lumen while elevating tissue and serum levels.

MATERIALS AND METHODS

Animals

The Animal Care and Use Committee of the University of Wisconsin-Madison and Middleton Veterans Administration Hospital, Madison approved all animal experimental protocols. Male Institute of Cancer Research (ICR) mice were purchased through Harlan (Indianapolis, IN) and housed in an American Association for Accreditation of Laboratory Animal Care-accredited conventional facility on the V.A Williamson Hospital Campus. Mice were acclimatized for one week in an environment controlled for temperature and humidity with a 12/12-hour light/dark cycle. Mice were housed 5 per covered/filtered box and fed *ad libitum* chow (LabDiet, PMI Nutrition International, St. Louis, MO) and water for 1 week prior to initiation of study protocol. After entry into study protocol mice were housed individually in metal cages with wire grid floors to prevent coprophagia and bedding ingestion.

Experimental Design

Experiment 1: Male ICR mice, ages 6 to 8 weeks, were randomized to Chow with an IV catheter (n = 11), PN (n = 11), intragastric (IG-) PN (n=11) via gastrostomy, or a complex enteral diet (CED, Nutren® 1.0, Nestle, Deerfield, IL) via gastrostomy (n=11). Animals were anesthetized by intraperitoneal injection of Ketamine (100 mg/kg) and Acepromazine (10 mg/kg). Animals with IV lines were cannulated in the vena cava via the right external jugular vein with a silicone rubber catheter (0.012" I.D./0.25" O.D.; Helix Medical, Inc., Carpinteria, CA). Gastric catheters were placed directly via gastrostomy. Catheters were tunneled subcutaneously, from

either the right jugular vein or gastrostomy site, over the back and exited mid tail. Mice were partially immobilized by tail fixation to protect the catheter during infusion. This technique does not induce significant physical or biochemical stress as was previously shown.[30]

Catherized mice were connected to infusion pumps and allowed recovery for 48 hours while receiving 4 mL/day saline (0.9%) and *ad libitum* chow (Agway Inc., Syracuse, NY) and water. A 2 day recovery was chosen because serum cytokines and corticosteroid levels induced by the surgical stress return to baseline, the airway and gut IgA response is over, and animals in all groups have resumed oral intake in normal amounts. Following the recovery period experimental diets were given. Chow mice received 0.9% saline at 4 mL/day as well as *ad libitum* chow and water throughout the study. PN and IG-PN fed mice received solution at 4 mL/day (day 1), 7 mL/day (day 2) and 10 mL/day (days 3-5) as well as *ad libitum* water throughout the study. The PN solution includes 6.0% amino acids, 35.6% dextrose, electrolytes, and multivitamins, with a non-protein calorie/nitrogen ratio of 126.1 (527.0 kJ/g Nitrogen). Since Nutren® contains 4.0% amino acids, 12.7% carbohydrates, 3.8% lipids, electrolytes, and multivitamins, with a non-protein calorie/nitrogen ratio of 130.4 (545.1 kJ/g Nitrogen), CED fed mice received the formula at 7 mL/day, 10 mL/day, and 14 mL/day (as well as *ad libitum* water) to provide a regimen which is isocalorical and isonitrogenous to the PN formula. Each formula meets the calculated nutrient requirements of mice weighing 25 to 30 g. [31]

After 5 days of feeding (7 days post-catherterization), mice were anesthetized by intraperitoneal injection of Ketamine (100 mg/kg) and Acepromazine (10 mg/kg). Serum samples were taken via exsanguination from a left axillary artery transection. During tissue sacrifice, portal vein serum was taken directly from the vessel. The small intestine was removed

and the lumen was washed with 20 ml Hanks Balanced Saline Solution (HBSS, Bio Whittaker, Walkersville, MD). The wash fluid was then centrifuged at 3,000 RPM for 10 minutes and 1 ml supernate was frozen at -80°C for analysis. Small intestinal tissue samples were taken by removing a 3 cm segment from the mid-distal and mid-proximal regions for ileum and jejunum samples, respectively. All tissues were frozen in liquid N_2 and stored at -80°C until processing and analysis.

Experiment 2: Male ICR mice, ages 6 to 8 weeks were randomized to Chow with intravenous saline (n = 22) or IV-PN (n = 22), as described above. After 5 days of feeding (7 days post-catherization) animals were randomized to sacrifice or surgical stress (11/group). Surgical stress was delivered as follows. The skin was disinfected using 75% ethanol and 2 wounds were then created. First, a 3.0-cm celiotomy incision was made and the small intestine was gently eviscerated and immediately returned to the peritoneal cavity. The wound was closed in 2 layers with 3 simple interrupted 4-0 silk sutures per layer. Second, a 1.5-cm ventral neck incision was made and blunt dissection carried down to the pretracheal plane. This wound was closed with a single layer of 2 simple interrupted 4-0 silk sutures. Eight hours later mice were again anesthetized (up to half original dose until righting reflex was lost) and sacrificed by exsanguination via left axillary artery transection. This time point was chosen due to previous observations that the maximal increase in respiratory and gut IgA levels in response to surgical stress occurred at 8 hours. Tissues were harvested as previously mentioned with the exception of portal vein serum due to limited splanchnic blood flow which prevented consistent sampling.

sPLA$_2$ Continuous Florescence Assay

Substrate Solution: Assay method followed previously published techniques by Tsao FHC et al [32], with minor modification of substrate preparation. Breifly, substrate was prepared by dissolving phosphotidylglycerol (Sigma, St. Louis, MO) in chloroform (2 mg/ml) and 1 ml aliquots were stored in glass vials with organic resistant cap at -20°C. 10 ul of Bis-BODipy FL C11-PN (Molecular Probes, Eugene, OR) fluorescently labeled probe was added to each 1 ml aliquot of dissolved phosphotidylglycerol. The chloroform was evaporated from the mixture under a stream of nitrogen gas until dry. Then, the chloroform free phospholipids were re-dissolved in 1 ml ethanol (100%) instead of Sucrose/Tris buffer (1ml; 0.25 mol/l sucrose, 50 mmol/l Tris-HCL, 0.02% sodium azide), modified to enhance assay sensitivity. The substrate solution was vortexed thoroughly and stable for up to a month when stored at -20°C.

Sample Preparation and Analysis: Assay samples were produced by mixing 987 uL Tris-HCL buffer, 10 uL substrate solution, and 3 uL sample to disposable glass culture tubes. Assay blanks were produced by mixing 990 uL 0.01 mol/l Tris-HCL buffer (pH 7.4) and 10 uL substrate solution into disposable glass culture tubes. Culture tubes were stored on ice during preparation. Then, triplicate volumes of 300 uL were aliquoted from the culture tubes to wells in white polystyrene 96-well microplates (Porvair PS White, Perkin Elmer, Norwalk, CT). Microplates were placed in temperature controlled (30°C) microplate reader (Perkin Elmer) and attached to a luminescence Spectometer LS50B (PerkinElmer). Florescence intensity of each well was recorded every 20s for 90 cycles at 488 nm excitation (excitation slit: 2.5 nm) and 530 nm emission (emission slit: 5.0 nm). To confirm calcium dependent sPLA2 activity, samples were also run with EGTA-Buffer (0.01 mol/l Tris-HCL (pH 7.4) containing 10 mmol/l Ca^{+2} and

20 mmol/l EGTA) which contains ample EGTA for complete calcium sequestration. After the reactions reached equilibrium temperature, the reaction curve was fit to a second-order polynomial equation and the first-degree coefficient was taken as the initial rate of reaction (expressed as change in FI/min/uL sample). Blank wells containing only substrate and buffer were used to find coefficient rates determined as background activity.

Western Blot for sPLA2-IIA

The amount of solubilized protein from each tissue homogenate was determined using the Bradford assay method. 10 ug of tissue homogenate protein, 4 uL of serum, or 4 uL of luminal wash fluid were denatured at 95°C for 10 minutes with sodium dodecylsulfate and beta-mercaptoethanol and separated in 4-15% agarose gel by electrophoresis at 150 V for 40 minutes at room temperature. Proteins were transferred to polyvinylidene fluoride membrane using tris-glycine buffer plus 20% methanol at 80 V for 35 minutes at 4°C. Membrane was blocked with 5% nonfat dry milk prepared in TBS-Tween for 1 hour at room temperature with constant agitation. Membranes were incubated with primary antibody, mouse anti-human sPLA$_2$-IIA (sc-58363, Santa Cruz Biotechnology, CA) duluted 1:1,000 over night at 4°C with constant agitation. Membranes were washed and incubated with stabilized goat anti-mouse IgG-HRP conjugate (sc-2005, Santa Cruz Biotechnology, CA) diluted 1:20,000 for 1 hour at room temperature with constant agitation. After washing, membranes were incubated with HRP substrate (Super Signal West Femto maximum sensitivity substrate; Pierce, Rockford, IL) for 5 minutes and bands were detected using photographic film. Molecular weight bands of 14-16 kDa were used to verify sPLA$_2$ concentration.

IgA Antibody Quantitative Analysis

IgA concentration from the SI luminal fluid was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (BD Biosciences, Bedford, MA) were pre-coated with 50 µl of a 10 µg/mL goat anti-mouse IgA, α-chain specific (Sigma-Aldrich, St Louis, MO) in 0.1 mol/L coating buffer (0.1M carbonate-bicarbonate, pH 9.6), and incubated overnight at 4 ^{o}C. Plates were washed 3 times and blocked with 100 µl/well of 1% bovine serum albumin in Tris-buffered saline with 0.5% Tween-20 solution for 1 hour at room temperature. SI luminal fluid samples were diluted (diluted 1:100) and 100 ul of diluted sample or IgA standards (seven 2-fold dilutions, from 1,000 → 7.8 ng/mL, Sigma-Aldrich) were added to respective wells and plates were incubated for 1 hour at room temperature. Plates were washed 3 times and 100 µL of a 1:500 dilution of the secondary antibody, goat anti-mouse IgA, α-chain specific-HRP conjugate (Sigma-Aldrich) were added to each well and incubated for 1 hour at room temperature. Plates were washed 3 times, and 100 µL of the substrate solution (H_2O_2 and *o*-phenylenediamine) were added for 7 minutes at room temperature. The reaction process was stopped by the addition of 50 µL of 2N H_2SO_4 and the absorbance read at 490 nm in a *Vmax* Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). SI luminal fluid IgA concentrations were calculated by plotting their absorbance values on the IgA standard curve, which was calculated using a 4-parameter logistic fit with SOFTmax PRO software (Molecular Device, Sunnyvale, CA).

Statistical analysis

Experimental values were compared using analysis of variance (ANOVA) and Fisher protected least significance difference (PLSD) corrected for multiple comparisons, with $\alpha = 0.05$ (Statview 5.0.1, SAS, Cary, NC). Serum vs Portal vein sPLA2 were analyzed with students paired T-test in each animal, respectively. Numerical results are presented as mean $\pm$ standard deviation of the mean.

RESULTS

Experiment 1: Route and type of feeding

Intestinal luminal sPLA$_2$ activity

CED (2.51 ± 0.53, $p = 0.0001$), IG-PN (3.1 ± 0.79, $p = 0.0002$), and PN (1.37 ± 0.83, $p = 0.0001$) significantly reduced SI luminal fluid sPLA2 activity (Fl/min/μL) compared to Chow (4.44 ± 0.96). PN significantly lowered SI luminal fluid sPLA$_2$ activity compared to CED ($p < 0.002$) or IG-PN ($p < 0.0001$). CED and IG-PN were statistically similar ($p = 0.09$) [Figure 1].

Serum and tissue sPLA$_2$ activity

Compared with Chow (4.46 ± 0.85), serum sPLA$_2$ activity was lower in CED (3.83 ± 0.7, $p = 0.042$), IG-PN (2.75 ± 0.37, $p < 0.0001$), and PN (3.3 ± 0.79, $p = 0.0004$). Compared with CED, IG-PN serum sPLA$_2$ activity was significantly lower ($p = 0.0008$) while PN failed to reach significance ($p = 0.08$). IG-PN and PN were not significantly different ($p = 0.07$) [Figure 2]. Portal vein serum sPLA$_2$ activity was significantly greater than systemic serum ($p = 0.04$) in the combined groups. Subgroup analysis of portal vs. systemic serum sPLA$_2$ activity showed that IG-PN and PN reached statistical significance (IG-PN: portal vein 2.53 ± 0.54 vs. systemic $1.99 \pm$

0.66, p < 0.001; PN: 3.07 ± 0.93 vs. 2.29 ± 0.82, p = 0.005) with no significant difference in the chow or CED groups (Chow: 3.924 ± 1.2 vs. 3.66 ± 1.39, p = 0.18; CED: 3.57 ± 0.79 vs. 3.3 ± 1.27, p = 0.09). Neither Jejunum nor Ileum tissue $sPLA_2$ activity was significantly affected by route of feeding.

Experiment 2: Stress and route of nutrition

Intestinal fluid $sPLA_2$ activity and IgA response

Consistent with Experiment 1, SI luminal fluid $sPLA_2$ activity (Fl/min/μL) in PN was significantly lower than Chow (2.47 ± 1.3 vs. 7.71 ± 2.1, p < 0.0001). Following stress, PN luminal fluid $sPLA_2$ activity remained low and unchanged (2.47 ± 1.3 vs. 3.4 ± 1.8, p = 0.3) and remained significantly lower than Chow at baseline (3.4 ± 1.8 vs. 7.71 ± 2.1, p < 0.0001). Stress significantly decreased SI luminal fluid sPLA2 activity in Chow (7.71 ± 2.1 vs. 3.5 ± 2.4, p < 0.0001) [Figure 3]. As previously reported, after stress SI luminal fluid IgA concentrations (ng/ml) significantly increased in Chow (209.3 ± 91 vs 404.7 ± 248, p = 0.0025) while a non-significant elevation was observed in PN (95.5 ± 33 vs 181.9 ± 66, p = 0.18). This slight elevation was due to a single outlier in the PN group. The level of IgA in Chow following stress was significantly greater than in PN at baseline (p < 0.0001) or PN following stress (p = 0.001) [Figure 4].

Serum and tissue sPLA2 activity

Serum $sPLA_2$ activity was significantly reduced in PN (3.06 ± 0.76) compared with Chow (4.19 ± 0.74, p < 0.03). Serum $sPLA_2$ activity remained unchanged in Chow (4.19 ± 0.74 vs. 4.41

± 1) mice after stress but significantly increased in PN compared with baseline (3.06 ± 0.76 vs. 4.18 ± 1.6, p < 0.03). The serum sPLA$_2$ activity in PN following stress (4.18 ± 1.6) was similar to both Chow at baseline (4.19 ± 0.74, p = 0.98) and Chow after stress (4.41 ± 1, p = 0.63) [Figure 5]. Jejunum tissue sPLA$_2$ activity remained unchanged [Figure 6A] with stress in both groups. PN mice exhibited an increase in Ileum sPLA$_2$ activity following stress (3.95 ± 2.9 vs. 6.33 ± 4.3, p = 0.16) but this failed to reach statistical significance [Figure 6B].

Western Blot

sPLA$_2$-IIA iso-form protein concentration determined with western blot was found to be similar to florescent activity measured via continuous florescence assay. These results are shown as sample gels with respective florescent activity results.

DISCUSSION

Both enteral and parenteral nutrition prevent progressive malnutrition in critically ill and critically injured patients compared with starvation, but PN is associated with an increase in infectious complications and multiple organ dysfunction. Although these PN-induced vulnerabilities are multi-factorial, both decreased mucosal defenses and increased tissue and systemic inflammation likely contribute to the observed morbidity. The implicated etiologies of increased infections include decreases in GALT cells, IgA production & transport; increases in bacterial virulence; and alterations in other factors such as GI permeability. Increases in tissue and serum sPLA$_2$ have been implicated in inflammatory regulation of eicosinoid production and neutrophil priming. sPLA$_2$ also plays a key role in innate defense of the small intestinal mucosa

as one of the intraluminal bactericidal defensin-related molecules. This work focused on this

molecule in experiments designed to investigate two questions. The first experiment explored

effects of altering route and complexity of diet on basal serum, tissue and luminal $sPLA_2$ activity.

The second experiment investigated the effect of induced surgical stress after enteral and

parenteral pretreatment in an established model of murine stress and injury which stimulates an

adaptive immune response in the form of increases in airway and intestinal IgA. This adaptive

immune response provides a marker of adaptive immune integrity in these experiments. Both

experiments analyzed $sPLA_2$ activity in the small intestine luminal fluid to evaluate potential

bactericidal activity in the small intestinal and its potential role as a regulator of inflammation in

the serum and tissue.

The first experiment proved consistent with our hypothesis that a decrease in enteral

stimulation (DES), achieved by reducing complexity of the enteral diet from chow to CED to IG-

PN to complete lack of enteral stimulation with PN, reduces gut mucosal defenses. PN resulted

in significant suppression of both IgA and $sPLA_2$ activity in the small intestine luminal fluid

compared with any enteral stimulation. The IgA changes are identical to our previously

published work (and show the reproducibility of our model) while suppression of $sPLA_2$ activity

in the intestine luminal fluid with unchanged tissue levels in jejunum or ileum suggests that

DES/PN decreases $sPLA_2$ production, secretion, or both producing a previously unrecognized

loss of an innate mucosal defense. Since the source of luminal $sPLA_2$ appears to be the Paneth

cell, this cell is the likely source of the defect. The suppressed $sPLA_2$ activity also correlates with

our previous studies showing that DES/PN reduces IgA protective mechanisms by reducing

levels of Th2 IgA-stimulating cytokines in the GALT as well as levels of polymeric

immunoglobulin receptor (pIgR), the princpal IgA transport protein in the mucosa[7-10]. In agreement with these studies of mucosal immunity, the degree of enteral stimulation correlates with sPLA$_2$ activity with Chow levels greater than CED and/or IG-PN, and all three greater than PN. Together these data support our hypotheses that DES/PN reduces bactericidal activity and bacterial binding within the gut, through inhibition of sPLA$_2$ and IgA, respectively. The effects of these DES/PN alterations of this defensin-related molecule upon the microflora environment remain unknown, but other work shows that lower sPLA$_2$ activity correlates with reduced bactericidal activity. Recent work in our laboratory (unpublished) demonstrates that intestinal sPLA$_2$ obtained from PN fed mice is much less bactericidal than sPLA$_2$ obtained from Chow fed mice despite equal concentrations of each. Therefore both the concentration and the form of sPLA$_2$ may be critical in controlling the bacterial population.

The second experiment showed that surgical stress significantly elevates systemic sPLA$_2$ after PN but not Chow feeding. This change in serum sPLA$_2$ is consistent with an acute illness [23,24] and further supports PN induced vulnerability to systemic inflammation. The second experiment also showed that surgical stress did not affect the already low luminal fluid sPLA$_2$ activity after PN, but significantly suppressed sPLA$_2$ activity in chow mice. The change in intestinal tissue levels implicates a decrease in sPLA$_2$ production. However, this may have been compensated for in Chow mice since another gut mucosal defense molecule, IgA, increased in the lumen as sPLA$_2$ levels dropped after injury. No significant elevation in IgA occurred in PN mice. One possible reason for the down regulation of sPLA$_2$ after injury in chow (and in all PN animals) is because of the potential for sPLA$_2$ to damage the host mucosa. Recent work by Dial *et al* demonstrated that neutralization of sPLA$_2$ in the small bowel reduced mucosal barrier

degradation when animals were challenged with intra-peritoneal LPS, a stimulus which induces a rapid increase in luminal sPLA$_2$ [33]. The mucosa normally heals quickly after injury and a mucus layer secreted by goblet cells protects its surface. Injury and PN reduce the mucus layer possibly rendering the host mucosa more vulnerable to sPLA$_2$ injury. In this instance, IgA, which is not bioactive toward the host, can bind bacteria to prevent their mucosal attachment during injury and recovery. PN suppresses both sPLA$_2$ and IgA after surgical stress implying a loss of both adaptive and innate branches of normal mucosal defense.

Our data also allow a general speculation regarding the source of sPLA$_2$ in these experiments. While Paneth cells lining the lumen are likely the source of luminal sPLA$_2$, the increase in circulation and ileal tissue levels in PN following stress implies that Paneth cells are not the source of this response since stress had no effect on luminal fluid sPLA$_2$ activity. The likely source appears to be the GI tract tissue itself and/or the GI vascular system since sPLA$_2$ activity was higher in the portal vein serum than in systemic serum. Subgroup analysis showed that portal sPLA$_2$ activity exceeded systemic levels in PN and IG-PN mice while CED also almost reached significance. Chow differences failed to reach significance due to increased sample variation and relatively small numbers of animals. We speculate that this may be due to reduced splanchnic blood flow with DES/PN, resulting in slower portal-systemic mixing and greater sPLA$_2$ concentration differences. In the GI tract vasculature, sPLA$_2$ has been shown to 'prime' neutrophils in both animal models to produce an augmented inflammatory response to subsequent stress or injury. In humans, sPLA$_2$ has also been implicated in neutrophil priming [28]. Since we previously showed that DES/PN primes neutrophils as well, we were surprised that portal vein levels were lowest in PN fed mice. However, the reduced sPLA$_2$ activity may be a

protective response since PN increases expression of ICAM-1 and p-selectin [4,5] in the gut vasculature and increases the number of PMNs sequestered onto the gut endothelium for interaction with the tissue and their priming.

There are several limitations to this study. First, we did not directly measure alterations in inflammation in the vascular system and relied on previous work performed in our animal models of diet and injury. Second, we did not differentiate mucosal $sPLA_2$ from other tissue compartments, such as the endothelium. Finally, we did not analyze the bactericidal or structural changes in $sPLA_2$ that could be occurring with altered Paneth cell function. These experiments are currently underway and preliminary results suggest that both are occurring.

In summary, this study strengthens the evidence that DES/PN suppresses mucosal defenses of the small intestine. While the effects of PN upon adaptive immune mechanisms of the intestinal mucosa were extensively studied, to our knowledge this work is the first evidence of suppressed innate immunity with PN. While we did not show direct evidence of altered inflammation by $sPLA_2$ via eicosanoid profiles or PMN priming, we confirmed the intestine is a significant source of this molecule and that injury in PN fed animals induces significant elevation in circulating $sPLA_2$ consistent with PN-induced vulnerability to injury.

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Figure 1 .
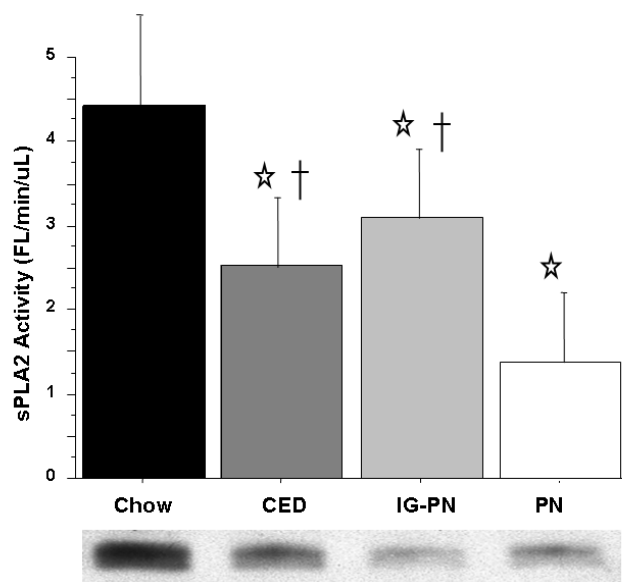
Intestinal Fluid sPLA2 activity. Intravenous parenteral nutrition (PN) and Intragastric parenteral nutrition (IG-PN) and complex enteral diet (CED) significantly suppress sPLA2 levels compared with Chow. PN sPLA2 activity was significantly less than CED and IG-PN. Representative western blot detected bands at ~14 kDA confirm sPLA2-IIA protein concentration in 4 uL of intestinal fluid. Values are means ± SD. *p<0.001 vs. Chow, †p<0.002 vs. PN.

Figure 2.
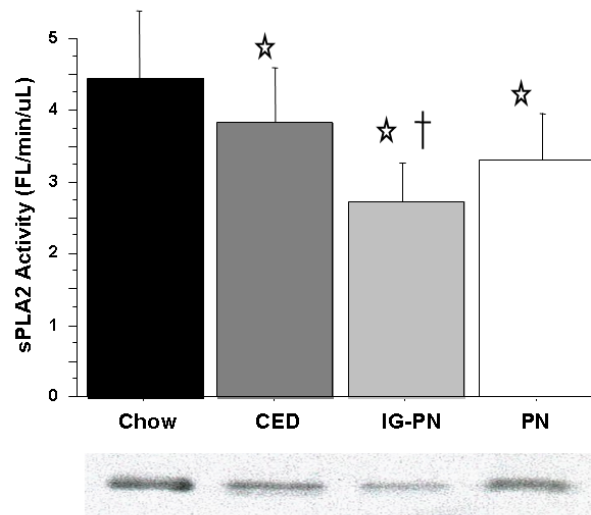
Serum sPLA2 activity. Intravenous parenteral nutrition (PN) and Intragastric parenteral nutrition (IG-PN) and complex enteral diet (CED) significantly suppress sPLA2 levels compared with Chow. Representative western blot detected bands at ~14 kDA confirm sPLA2-IIA protein concentration in 4 uL serum. Values are means ± SD. *p<0.05 vs. Chow, †p<0.001 vs. CED.

Figure 3.
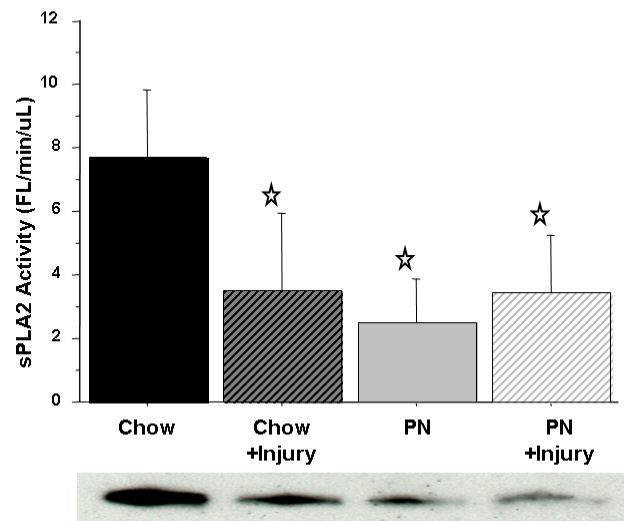
Intestinal Fluid sPLA2 activity. sPLA2 activity was significantly suppressed in Chow+Stress, Parenteral Nutrition (PN), and PN+Stress, compared with Chow. Representative western blot detected bands at ~14 kDA confirm sPLA2-IIA protein concentration in 4 uL of intestinal fluid. Values are means ± SD. *p<0.0001 vs. Chow.

Figure 4.
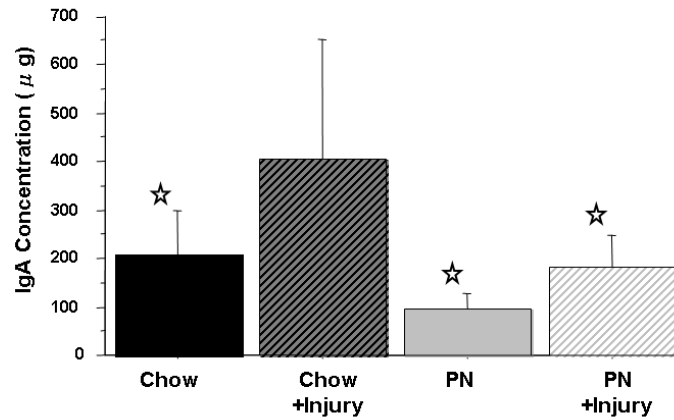
Intestinal Fluid IgA concentration. Parenteral Nutrition (PN) significantly suppressed IgA levels compared with Chow. Surgical Injury significantly increased IgA in Chow+Stress compared with Chow, while no changes were observed in PN+Stress compared with PN.
Values are means ± SD. *p<0.003 vs. Chow+Stress.

Figure 5.
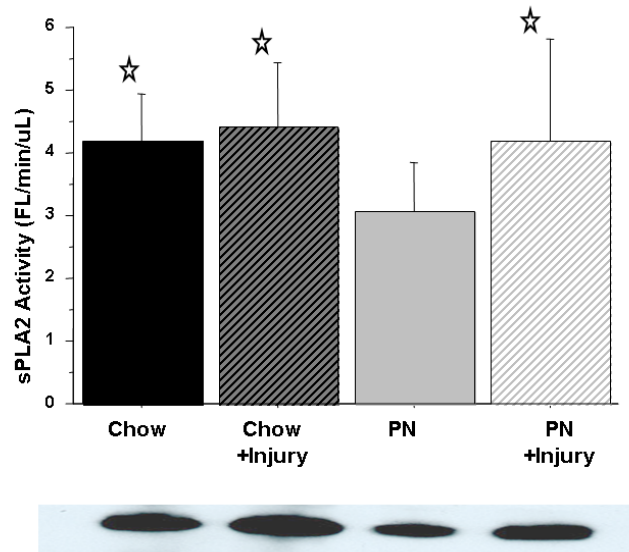
Serum sPLA2 activity. PN significantly suppressed sPLA2 activity compared with Chow. Surgical Injury significantly increased serum sPLA2 in PN+Stress, but not Chow+Stress, compared with respective baseline levels. Representative western blot detected bands at ~14 kDA confirm sPLA2-IIA protein concentration in 4 uL serum.
Values are means ± SD. *p<0. 03 vs. PN.

Figure 6A & 6B.
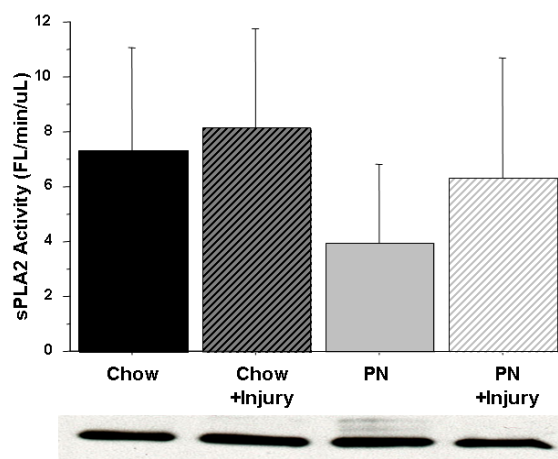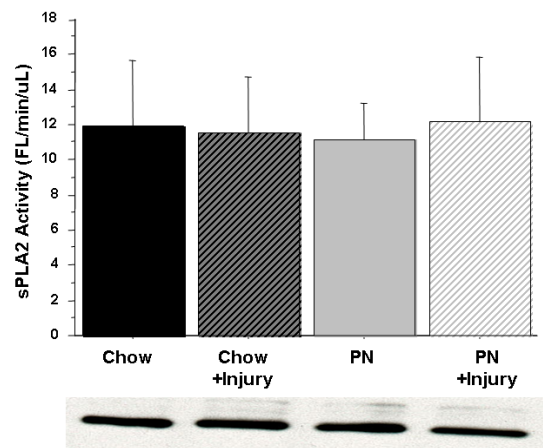
Small Intestine Tissue sPLA2 activity.
There were no effects on sPLA2 activity with Parenteral Nutrition (PN), Chow, or subsequent Surgical Injury in either group in the Jejunum (A) or Ileum (B) tissue. Representative western blot detected bands at ~14 kDA confirm sPLA2-IIA protein concentration in 10 ug total tissue protein.

Chapter 3
Parenteral Nutrition Suppresses the Bactericidal Response of the
Small Intestine

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ABSTRACT

Background: Parenteral nutrition (PN) increases infectious risk in critically ill patients compared with enteral feeding. Previously, we demonstrated that PN feeding suppresses the concentration of the Paneth cell antimicrobial protein secretory phospholipase A2 (sPLA$_2$) in the gut lumen. sPLA$_2$ and other Paneth cell proteins are released in response to bacterial components, such as lipopolysaccharide (LPS), and they modulate the intestinal microbiome. Since the Paneth cell protein sPLA2 was suppressed with PN feeding, we hypothesized PN would diminish the responsiveness of the small bowel to LPS through reduced secretions and as a result exhibit less bactericidal activity.

Methods: The distal ileum was harvested from ICR mice, washed, and randomized for incubation with LPS (0, 1, or 10 µg/mL). Culture supernatant was collected and sPLA$_2$ Activity was measured. Bactericidal activity of the ileum segment secretions was assessed against *P. aeruginosa* with and without a sPLA2 inhibitor at two concentrations, 100nM and 1µM. ICR mice were randomized to Chow or PN for 5 days. Tissue was collected for immunohistochemistry (IHC) and ileal segments were incubated with LPS (0 or 10 µg/mL). sPLA$_2$ activity and bactericidal activity were measured in secretions from ileal segments.

Results: The ileal segments responded to 10 ug/mL LPS with significantly greater sPLA$_2$ activity and bactericidal activity. The bactericidal activity of secretions from LPS stimulated tissue was suppressed 50% and 70%, respectively, with the addition of the sPLA2-inhibitor. Chow displayed greater sPLA$_2$ in the Paneth cell granules and secreted higher levels of sPLA2 than PN before and after LPS. Accordingly, media collected from Chow was more bactericidal than PN. IHC confirmed a reduction in Paneth cell granules after PN.

Conclusions: This work demonstrates that ileal segments secrete bactericidal secretions after LPS exposure and the inhibition of the Paneth cell antimicrobial protein sPLA2 significantly diminishes this. PN feeding resulted in suppressed secretion of the $sPLA_2$ and resulted in increased bacterial survival. This demonstrates that PN significantly impairs the innate immune response by suppressing Paneth cell function.

CLINICAL RELEVANCY

Prolonged PN administration is associated with increased incidence of infections. This work demonstrates that PN reduces the innate immunity response of the GI tract to LPS as measured by reduced secretions. This impairment results in less bactericidal activity towards *Pseudomonas aeruginosa*, a pathogen which contributes to infectious complications.

INTRODUCTION

Parenteral nutrition (PN) provides essential nutritional support to patients with contraindications to enteral feeding. Unfortunately, PN is associated with an increased risk of septic complications from intra-abdominal abscesses and pneumonia in critically ill trauma patients compared to patients fed enterally [1,2]. Increased susceptibility to infection following PN is linked to multiple factors including altered intestinal permeability, increased virulence of resident microbes, and alterations in acquired immune defenses manifested through gut associated lymphoid tissue (GALT) changes. GALT changes include fewer Peyer's patch and lamina propria lymphocytes, reductions in IgA-stimulating cytokines, and lower levels of respiratory and intestinal IgA levels[3,4]. The lower IgA levels provide less anti-bacterial and anti-

inflammatory protection for the host mucosa since IgA normally aggregates pathogens and

prevents their attachment to the mucosa. But innate immune mechanisms also protect the mucosa

though physical barriers such as a mucin layer produced by Goblet cell, maintenance of

enterocyte tight-junctions, and the secretion of innate antimicrobial molecules, the majority of

which are peptides and proteins produced by Paneth cells.[5,6] These molecules play a vital role in

preventing microbial colonization of the mucosa[7,8] and their loss is associated with spontaneous

inflammation of the small bowel[9].

We recently demonstrated that PN feeding with or without injury significantly decreases

the activity of one innate antimicrobial molecule, secretory phospholipase A2 ($sPLA_2$), in the gut

lumen[10]. This suggested that route of dietary administration affects Paneth cell production or

secretion, since this cell is the source of luminal $sPLA_2$. Paneth cells constitutively express

$sPLA_2$ and other antimicrobial proteins, such as lysozyme, RegIIIγ, and defensins or cryptidins

which are stored in intracellular granules before release into the gut lumen. $sPLA_2$-IIA is highly

expressed in Paneth cell granules and exhibits potent antimicrobial activity[11]. Its catalytic site

cleaves free fatty acids from the sn-2 position of phospholipid glycerol backbones, releasing free

fatty acids and lyso-phosphlipids. Due to the protein's cationic charge, $sPLA_2$ preferentially

attacks negatively charged phospholipids, such as phosphotidylethanolamine and

phosphotidylserine, which are found in high concentrations in bacterial cell membranes [12-13].

Accordingly, $sPLA_2$ has been shown to disrupt cell membranes in both gram-positive and gram-

negative bacterial strains, inducing bacterial cell death [6,11,14,15].

Although the effects of PN on adaptive gut and respiratory immunity through remodeling

of GALT have been studied, the innate gut responses following PN have not been explored

mechanistically. This work investigates the effect of route of nutrition on Paneth cell function by measuring LPS-induced sPLA$_2$ secretion and the resulting bactericidal function against *Pseudomonas aeruginosa* in an *ex vivo* small intestinal model. We also determined the bactericidal activity attributable to sPLA2 using an sPLA2 inhibitor, since several constitutively expressed antibacterial molecules are secreted in response to bacterial components [16]. We hypothesized that PN suppresses the intestinal capacity to respond to bacterial stimuli through reduced Paneth cell secretions resulting in suppressed bactericidal activity.

MATERIALS AND METHODS

Animals

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

Experimental Designs

Kinetic of sPLA$_2$-IIA secretion by the small bowel following LPS stimulation.

To quantify the dose responsiveness and kinetics of sPLA2 release from small bowel segments after LPS stimulation for subsequent experiments, chow (Rodent Diet 5001; LabDiet) fed mice (7 to 8 weeks old, n = 6) were anesthetized by intramuscular injection (ketamine 100 mg/kg and acepromazine maleate 10 mg/kg mixture) and euthanized by exsanguination prior to harvesting the small intestine (SI). The SI lumen was flushed with 20 mL of ice cold calcium-magnesium-free Hank's balanced salt solution (CMF-HBSS) (Bio Whittaker, Walkersville, MD) to collect the SI wash fluid. An additional 120 mL of ice cold CMF-HBSS was used to thoroughly rinse the tissue. The distal ileum was sectioned into six 2 cm segments, opened longitudinally, and placed in 24-well multidishes (Nunc, Roskilde, Denmark) containing 1 mL CMF-HBSS on ice. Segments were then randomized to receive Control LPS0 (PBS), LPS1 (1 µg/mL LPS from *Salmonella enterica*,(Cat L6511, Sigma, St. Louis, MO)) or LPS10 (10 µg/mL LPS,) in duplicate. Tissues were incubated at 37°C and culture media was collected at 0, 5, 10, 20, 40, 60 and 120 min. Collected culture media was centrifuged at 3000 rpm for 5 min at 4°C to remove cellular debris, passed through a 0.22 µm filter, and stored at -80°C until sPLA$_2$ analysis. The kinetics of sPLA$_2$ activity in culture media were examined by Continuous Fluorescence Assay at the time points specified. sPLA$_2$-IIA concentration was confirmed with Western blot. Analysis of bactericidal activity was performed on culture media from the 120 minute time point.

Bacteria Preparation and Bactericidal Assay

To determine bacterial activity, *Pseudomonas aeruginosa* (*P. aeruginosa*) was cultured overnight in a LB broth (Fisher Scientific, Fair Lawn, NJ) for 24 hr at 37°C. The culture was centrifuged at 2060 x g for 10 min at 4°C, washed, and resuspended in sterile CMF-HBSS. A

bacterial suspension was adjusted to 2×10^3 CFU /mL with CMF-HBSS. To evaluate

bactericidal activity, 10 μL (~100 CFU) of bacterial stock was combined with 200 μL culture

media or CMF-HBSS (Control) and incubated for 1 hr at 37°C. Surviving CFUs were determined

in duplicate by plating on LB agar plates (Fisher Scientific, Pittsburg, PA) under aerobic

condition at 37°C overnight in triplicate. CFUs were normalized to control plates in respective

experiments and results were expressed as bactericidal activity as measured by the reduction in

% CFUs.

Bactericidal Activity following sPLA2 inhibitor

To determine the contribution of sPLA2 to the bactericidal activity fluid released by the isolated

small bowel segments after LPS stimulation, wash fluid from the intestine of chow (Rodent Diet

5001; LabDiet) fed mice (8 weeks old, n = 4) were collected and prepared as described above

using CMF-HBSS (control) or LPS10 (10 μg/mL) for 60 min at $37°$C. The bactericidal assay

was performed on collected secretions with CMF-HBSS (control), LPS10, or LPS10 with the

addition an sPLA2-IIA inhibitor at two concentrations, 100 nM and 1 μM, (Cat. 525145, cyclic

(2-NaphthylAla-Leu-Ser-2-Naphthyl Ala-Arg)TFA, EMD Chemicals, Gibbstown, US). Each

treatment was inoculated with 10 uL (~100 CFU) bacteria for 60 min at $37°$C. Surviving CFUs

were plated in duplicate on LB plates and incubated overnight at $37°$C.

The effect of PN on small intestine sPLA$_2$ secretion and bactericidal function following LPS

stimulation

Male ICR mice (7 to 8 weeks old, n = 10) were randomized to the Chow (n = 5) or the PN (n = 5) group. Animals were anesthetized by intramuscular injection, weighed, and underwent placement of silicon rubber catheter (0.012-inch I.D./0.025-inch O.D.; Helix Medical, Inc., Carpinteria, CA) in the vena cava through the right external jugular vein. The catheter was tunneled subcutaneously over spine and existed at the midpoint of the tail. The animals were housed individually in metabolic cages with wire floors to prevent coprophagia and bedding ingestion and partially immobilized by tail restraint to protect the catheter during infusion. This technique has proven to be an acceptable method of nutritional support and does not produce physical or biochemical evidence of stress [17].

All mice were given chow and water *ad libitum* with normal saline (0.9%) infusion at 4 mL/day for 48 hr to allow surgical recovery as was previously measured by serum cytokines and normalized oral intake. Chow mice continued to receive intravenous saline (0.9%) at 4 mL/day with free access to chow (Rodent Diet 5001; LabDiet) and water throughout the study period. PN animals received PN solution at rates 4 mL/d (day 1), 7 mL/d (day 2) and 10 mL/d (day 3 to 5) because a graded infusion period was demonstrated to be necessary for the mice to adapt to the glucose and fluid loads. The PN solution contained 6.0% amino acids, 35.6% dextrose, electrolytes, and multivitamins, containing 1440 kcal/L and a non-protein calories/nitrogen ratio of 128:1. These values were calculated to meet the nutrient requirements of mice weighting 25 to 30 g [18].

After 5 days of feeding, mice were euthanized as described above. The small bowel was harvested, cleaned of mesenteric fat and vascular tissue, and the lumen was flushed with 20 mL of ice cold CMF-HBSS. This wash fluid was then centrifuged at 2000 xg for 10 min at 4°C and a

1 mL supernatant sample was stored at -80°C for sPLA$_2$ activity. Next, the intestinal lumen was

rinsed thoroughly with an additional 120 mL of ice cold CMF-HBSS. Ileum segments were

randomized to Control (PBS) or LPS10 (LPS 10 µg/mL) in 1 mL CMF-HBSS for 1hr at 37°C.

Culture media was centrifuged at 3000 rpm for 5 min at 4°C, passed through a 0.22 µm filter,

and stored at -80°C. Western blot was used to identify sPLA$_2$-IIA in culture media. sPLA$_2$

activity was determined in both the intestinal wash fluid and culture media and a bactericidal

assay was performed on the culture media from LPS stimulated tissues. Additional Ileum

segments were fixed for Immunohistochemistry of sPLA2-IIA.

Continuous fluorescent assay for sPLA$_2$ Activity

Fluorescent assay for sPLA$_2$ activity was performed as described previously by *Tsao et al* [19],

with some modification to substrate preparation[10]. This assay uses a specific probe, Bis-BODipy

FL, which is designed to fluoresce when the Sn2 position of the phospholipid glyceraldehyde

backbone is cleaved. This method was established as a high throughput method to rapidly

analyze sPLA2 activity. Briefly, substrate was prepared by mixing 10 uL Bis-BODipy FL C11-

PN (Molecular Probes, Eugene, OR) in a 1 mL aliquot of phosphatidylglycerol (Sigma, St.

Louis, MO) dissolved in chloroform (2 mg/mL) and evaporated under nitrogen. The chloroform-

free phospholipids were re-dissolved in 100% ethanol and used as substrate. The ethanol

substrate solution was stable for month stored at -20°C. The assay reaction mixture was prepared

in a glass tube on ice that contained 10 µL of substrate ethanol solution (20 µg of phospholipids)

and 12.3 µL of sample in a final volume of 1 mL that was made up with buffer of 0.01 M Tris-

HCl (pH 7.4) containing 10 mM Ca^2.An aliquot of 0.3 mL of the reaction mixture was promptly

transferred in triplicate to the wells of a white polystyrene microplate (Porvair PS White, PerkinElmer Instruments, Norwalk, CT). The microplate was placed in a temperature-controlled (30°C) microplate reader attached to a PerkinElmer Luminescence Spectrometer LS50B. The fluorescence intensity (FI) in each well was recorded every 10 sec for 60 cycles at 488 nm excitation (excitation slit: 2.5 nm) and 530 nm emission (emission slit: 5.0 nm).). To confirm calcium dependent sPLA2 activity, samples were also run with EGTA-Buffer (0.01 mol/l Tris-HCL (pH 7.4) containing 10 mmol/l Ca^{+2} and 20 mmol/l EGTA) which contains ample EGTA for complete calcium sequestration. After the reactions reached equilibrium temperature, the reaction curve was fit to a second-order polynomial equation and the first-degree coefficient was taken as the initial rate of reaction (expressed as change in FI/min/uL sample). Blank wells containing only substrate and buffer were used to find coefficient rates determined as background activity.

Western blot for sPLA2 in the tissue culture media

10 uL of tissue culture media was denatured at 95°C for 10 min with sodium dodecylsulfate and β-mercaptoethanol and separated in a 4-15% polyacrylamide gel (Ready Gel, Bio-Rad Laboratories, Hercules, CA) by electrophoresis at 150 V for 42 min. with molecular weight markers (Dual Color, Precision plus protein standards, Bio-Rad Laboratories). Proteins were then transferred to polyvinylidene fluoride membranes using a Tris-glycine buffer plus 20% methanol at 80 V for 50 min. Membranes were blocked with 5% nonfat dry milk prepared in Tris-buffered saline with 0.5% Tween-20 (TBS-Tween) for 1 hr with constant agitation. Membranes was incubated with the anti-group II sPLA$_2$ antibody (sc-14468, Santa Cruz Biotechnology Inc.,

Santa Cruz, CA) diluted 1:100 overnight at 4ºC with agitation. Then membranes were washed

and incubated with donkey anti-goat goat IgG-HRP conjugate (sc-2020, Santa Cruz

Biotechnology) diluted 1:5000 for 1 hr at room temperature with agitation. Following washing,

the membranes was incubated with the ECL reagent (Super Signal West Femto Maximun

Sensitivity Substrate, Pierce Biotechnology, Rockford, IL) for 5 min at room temperature and

bands were exposed to photographic film within 30 sec.

Quantification of sPLA2 in Chow and PN Ileum segments

To quantify the concentration of sPLA2 in Paneth cell granules, we performed IHC on the ileum

segments following Chow or PN feeding. Samples were fixed in 4% paraformaldehyde overnight,

transfer to 70% ethanol, and stored at 4 Cº until processing. Samples were processed in a Tissue-

Tek V.I.P tissue processor which included the following steps: 70% ETOH, 45 min; 80% ETOH,

45 min; 95% ETOH, 2x1h; 100% ETOH, 2x1h; Xylene, 2x1h; Paraffin MP 60ºC (Surgipath,

Richmond, IL), 2x45 min; Paraffin, 2x1h. Samples were then embedded in paraffin, sectioned at

5 µm with a microtome, and placed on Adhesive Coated Slides (White Aminosilane, Newcomer

Supply, Madison, WI). Paraffin was melted by placing slides in 60 C for 35 mins. Slides were

placed in xylene to remove remaining paraffin and rehydrated in graded alcohol baths. Antigen

retrieval was performed by boiling slides in 10mM sodium citrate buffer (pH 6.0). Samples were

outlined using a pap-pen. 10% BSA-PBS was used to block non-specific binding for 1 hour.

Samples were then incubated with primary antibody (group II sPLA2 (G-15) goat polyclonal IgG,

sc-14468, Santa Cruz Biotechnology) overnight in 1% BSA-PBS at 4 C in a humidity chamber.

Remaining solution was tapped off and samples were incubated in the dark with secondary

antibody (Alexa Fluor 594, donkey anti- goat IgG, 2mg/mL, A11058, Invitrogen, Grand Island, NY) for 30 mins in 1% BSA-PBS at room temperature. DAPI (P36935, Invitrogen) was placed on each slide before covering to image nuclei. After imaging, densitometric measurements of sPLA2 intesity were quantified in 12 individual crypts per animal using NIH ImageJ software.

Statistical analysis

The data are expressed as means ± standard error of the mean. Statistical significance was determined using ANOVA with Fisher's protected least significant difference *post hoc test* or Student's *t*-test. Bactericidal activity was measured by normalizing treatment group Colony Forming Units (CFUs) to the control group using paired analysis in each respective experiment. Treatment group CFUs were compared using ANOVA. Differences were considered to be statistically significant at *p<0.05*. All statistical calculations were performed with StatView (Abacus Concepts, Berkeley, CA).

RESULTS

Kinetics of sPLA$_2$-IIA secretion following LPS Stimulation

Increased sPLA$_2$ activity occurred in all treatments, reached a peak at 60 minutes, and remained stable through 120 minutes [Figure 1A]. At 120 minutes, LPS10 stimulated significantly more sPLA$_2$ activity (1.93 ± 0.24 Fluorescence/min/µL) than LPS0 (1.04 ± 0.19, p < 0.05) but not LPS1 (1.26 ± 0.32, p = 0.08) with no significant differences between LPS0 and LPS1 (p = 0.57). All sPLA$_2$ activity was absent in LPS10 culture medium with EGTA confirming that the activity measured in the treatments was calcium dependent, excluding

calcium-independent cytosolic PLA_2 as the source. Western blot for $sPLA_2$-IIA in secretions also showed no change between LPS0 and LPS1, while LPS10 demonstrated an increased sPLA2 concentration consistent with the quantified activity [Figure 1B].

Bactericidal activity of tissue culture medium

Both LPS0 ($p < 0.08$) and LPS10 ($p < 0.003$) samples demonstrated bactericidal activity compared to control HBSS [Figure 2]. There was no significant difference between LPS0 and LPS10.The bactericidal activity in LPS0 was consistent with baseline levels of $sPLA_2$ secretion observed in un-stimulated tissue.

Bactericidal Activity following sPLA2 inhibitor

Consistent with the kinetics, LPS10 induced significantly greater bactericidal activity than control HBSS samples [Figure 3]. Compared with LPS10 alone, addition of sPLA2-IIA inhibitor reduced bactericidal activity at both inhibitor concentrations but only the 1μM concentration reached statistical significance ($p = 0.03$). There was no statistical difference in bactericidal activity between the control HBSS and either inhibitor doses (100nM : $p = 0.18$ and 1μM : $p=0.35$) or between the two inhibitor concentrations ($p = 0.6$).

The effect of Chow and PN on secretion and bactericidal function of $sPLA_2$-IIA
Body Weight**:**

There were no significant differences in initial body (Chow vs PN: 34.3 ± 2.3 vs. 33.1 ± 1.8 grams, p = 0.69) or weight change between Chow and PN groups (-3.4 ±1 vs. -5.5 ±1.1 grams, p = 0.20).

sPLA$_2$ activity of SIWF:

Consistent with our previous findings[10], SI wash fluid sPLA$_2$ activity was significantly lower in PN than Chow (2.6 ± 0.5 vs. 11.9 ± 1.1 FL/min/μL, $p < 0.001$).

sPLA$_2$ activity of culture medium:

At baseline (0 μg/mL LPS), chow fed mice demonstrated greater sPLA2 activity (0.9 ± 0.1 FL/min/μL) than PN (0.45 ± 0.1, p = 0.01) [Figure 4]. Following LPS stimulation (10 μg/mL LPS), Chow sPLA2 activity significantly increased compare to baseline (1.3 ± 0.15 vs. 0.9 ± 0.1, p = 0.02), while PN was non-significantly increased due to variation in tissue responsiveness (0.8 ± 0.3 vs. 0.45 ± 0.1, p = 0.3). However, despite LPS stimulation, PN sPLA2 activity (0.8 ± 0.3) only reached baseline Chow levels (0.9 ± 0.1, p = 0.5). Western blot results demonstrated sPLA2-IIA concentrations consistent with quantified sPLA2 activity in respective treatments [Figure 4B].

Bactericidal activity of culture medium:

Bactericidal activity in Chow ($17 \pm 3\%$) culture media was significantly greater than control HBSS ($0 \pm 4\%$, $p < 0.01$) or PN ($5 \pm 4\%$, $p < 0.04$) [Figure 5]. PN failed to reach significance over control HBSS (p = 0.40).

Immunohistochemistry of sPLA2-IIA in Ileum:

sPLA2-IIA was clearly visible in the Paneth cell granules, located at the base of the intestinal

crypts [Figure 7]. Densitometric analysis of sPLA2-IIA intensity was significantly reduced in PN

(386.7 ± 180.5 Relative intensity (RI), $p = 0.01$) compared with Chow (3118.0 ± 1017 RI)

[Figure 6], consistent with decreased luminal sPLA2 activity and reduced tissue secretions

following LPS challenge in PN tissue.

DISCUSSION

The intestinal mucosal surface constitutes a dynamic interface between the host and

microbiome that plays essential roles in human health by enhancing nutrient metabolism,

promoting immune development, and limiting pathogen colonization. Despite the dense and

diverse community of microorganisms, both the host and bacteria benefit illustrating the highly

effective mechanisms for monitoring and regulating bacterial interactions with the intestinal

mucosal interface. During illness or following injury, this balance may be impaired due to

interruptions in nutrient intake, the use of parenteral nutition, intestinal ischemia and acidosis,

antibiotics and other aspects of clinical care and the clinical environment.

Recently we observed that PN with lack of enteral stimulation reduced the activity of

$sPLA_2$ in small intestinal wash fluid both prior to and following surgical stress compared to chow

fed mice [10]. $sPLA_2$ is an innate immune molecule produced by Paneth cells, which are secretory

epithelial cells located at the base of intestinal crypts throughout the intestine that produce a

variety of bactericidal peptides and proteins, including human α-defensins or murine cryptidins,

lysozyme, Reglll-γ, in addition to sPLA$_2$. These agents remain stored in intracellular granules before their release into the lumen.[6]. Production of Paneth cell granular products is not dependent on microbial presence, but secretion is regulated in part through response to binding of pathogen-associated molecular pattern (PAMP) to innate receptors, such as extracellular Toll-Like Receptors (TLRs) and intracellular Nucleotide- Oligermerization Domains (NODs). The innate receptors respond to various bacterial antigens, including Lipopolysaccharide (LPS) - a well conserved gram-negative cell wall component - that binds TLR-4.

This work focused on sPLA$_2$ activity and bactericidal activity of secretions from small intestinal segments following LPS-stimulation. Then, we analyzed these parameters following Chow and PN feeding regimens. We first established the kinetics of sPLA$_2$ secretion from intestinal tissue following LPS-stimulation using two LPS doses, which was consistent with others work showing release of innate bactericidal molecules after LPS stimulation[16,20]. We demonstrated that sPLA$_2$ secretion from *ex vivo* tissue segments reaches maximal levels after 60 minutes under our experimental conditions, and that 10 µg/mL LPS significantly stimulates sPLA2 compared to 0 or 1 µg/mL LPS. Interestingly, there was no measurable increase between 0 and 1 µg/mL LPS, perhaps due to the constant bacterial presence in the bowel and a threshold of tolerance. We also observed a baseline level of sPLA$_2$ secretion, even in the absence of experimental LPS-stimulation. We then determined the bactericidal activity against *Pseudomonas aeruginosa* and found secretions from LPS-stimulated tissue contained the most activity, but un-stimulated tissue still possessed activity consistent with baseline secretions.

To determined the contribution of sPLA2 to the observed bactericidal activity, we added two concentrations, 100 nM and 1µM of a potent sPLA2 inhibitor, cyclic (2-NaphthylAla-Leu-

Ser-2-Naphthyl-Ala-Arg)TFA, to the secretions obtained from LPS10 stimulated tissue. The inhibitor reduced bactericidal by approximately half, 54%, at a concentration of 100 nM, and approximately 70% of bactericidal activity was lost with 1 μM. This confirmed that sPLA2 contributed a large percentage of bactericidal activity against *Ps. Aeruginosa*. sPLA2 works synergistically with a myriad of molecules within the gut lumen including lysozyme, Reg-IIIγ, and angiogenin-4 to modulate bacteria. We did not measure the contribution of these individual molecules but such studies are currently underway.

After establishing LPS dose and time points, we examined the effect of PN with the lack of enteral stimulation on the intestinal sPLA$_2$ responsiveness to LPS and the associated bactericidal function compared with chow feeding. Our previous showed PN feeding reduced sPLA$_2$ activity in the small intestinal wash fluid[10]. Consistent with those data, we demonstrated that PN blunted the sPLA$_2$ response to LPS by intestinal tissue potentially indicating reduced levels of sPLA2 in the Paneth cell granules, a reduced ability to release granules, or both. We addressed both possibilities using immunohistochemistry (IHC) and the LPS stimulation technique. IHC demonstrated significantly reduced sPLA2-IIA expression within the Paneth cell granules following PN feeding [Figure 6] indicating that sPLA2-IIA production, and perhaps production of other constitutively expressed antimicrobial proteins, is down-regulated following reduced enteral feeding, such as occurs with PN. The significantly suppressed bactericidal activity from PN secretions, even despite LPS stimulation, confirms the relevance of these findings.

These data confirm our hypothesis that reduced enteral stimulation suppresses innate mucosal immunity in the small bowel and provide the first mechanistic experimental work into

the effect of dietary route on innate mucosal responsiveness to bacterial components. The impaired innate mucosal defense following PN suggests an impaired ability to influence intestinal microbiota.

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Figure 1A.
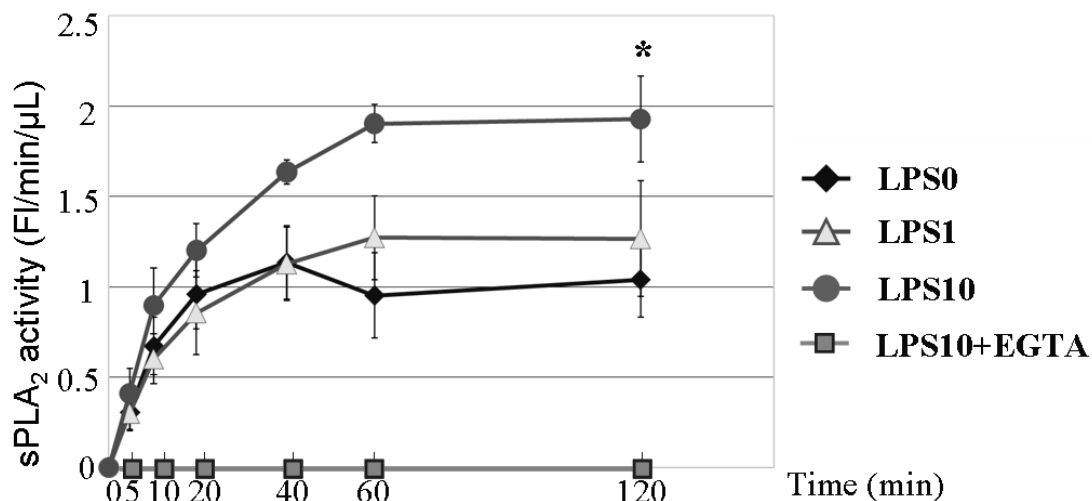
 sPLA2 release following LPS Stimulation of Intestinal Segments. Kinetics of $sPLA_2$ activity (FL/min/μL) in culture media following LPS stimulation at LPS doses of 0 (LPS0), 1 (LPS1), and 10 μg/mL (LPS10). sPLA2 activity reached a plateau at 60 minutes and remained constant through 120 minutes. At 120 minutes LPS10 was significantly greater than LPS0 but failed to reach significance vs LPS1. LPS1 did not significantly differ from LPS0.
* $p < 0.05$ vs. LPS0

Figure 1B.
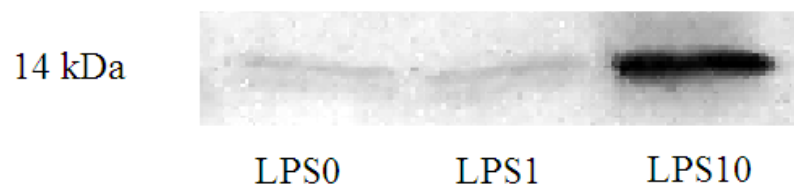
Representative sPLA2-IIA western blot of tissue culture media after LPS stimulation. LPS0 and LPS1 did no differ, while LPS10 showed an increase in sPLA2-IIA concentration consistent with the observed activity. Bands are 14 kDa molecular weight.

Figure 2.
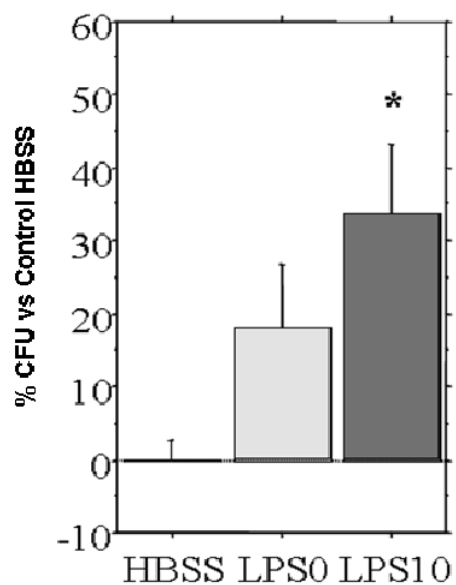
 Bactericidal Activity of tissue secretions ± LPS Stimulation. Bactericidal activity against *Ps. Aeruginosa* from culture media unstimulated, LPS0 (0 µg/mL LPS), and LPS10 (10 µg/mL LPS) compared with HBSS control. LPS10 had significantly greater bactericidal activity than HBSS control, while LPS0 failed to reach significance ($p < 0.08$). * $p<0.05$ vs HBSS

Figure 3.
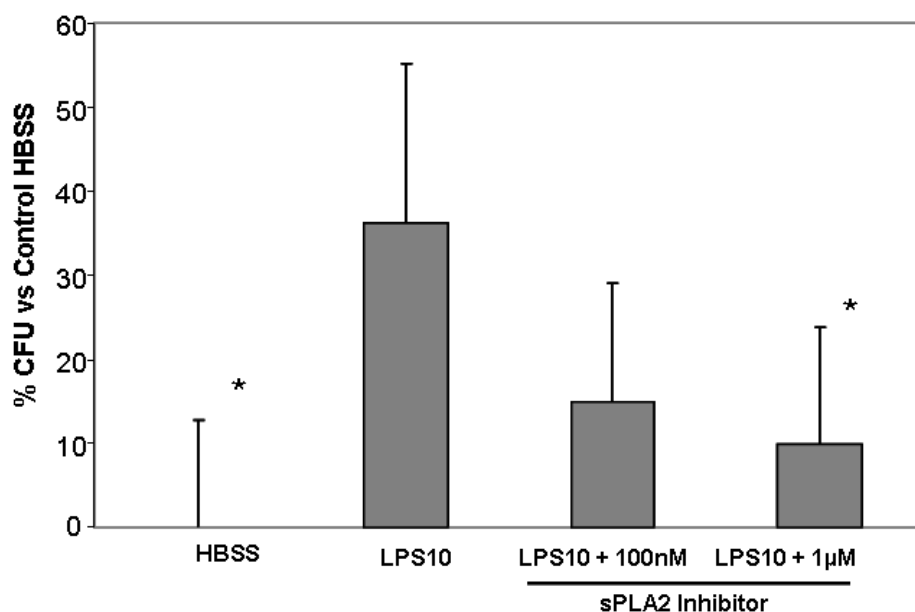
Bactericidal Activity of LPS stimulated tissue secretions with sPLA2 Inhibitor. Bactericidal Activity of culture media against *Ps. Aeruginosa* with an sPLA2 Inhibitor. LPS10 (10 μg/mL LPS) demonstrated significant bacterial killing compared with the HBSS control. The sPLA2 inhibitor 100 nM suppressed killing non-significantly ($p = 0.07$), while the higher concentration 1 μM significantly suppressed bacterical activity. There was no change between inhibitors and HBSS control. * $p<0.05$ vs LPS10

Figure 4A.
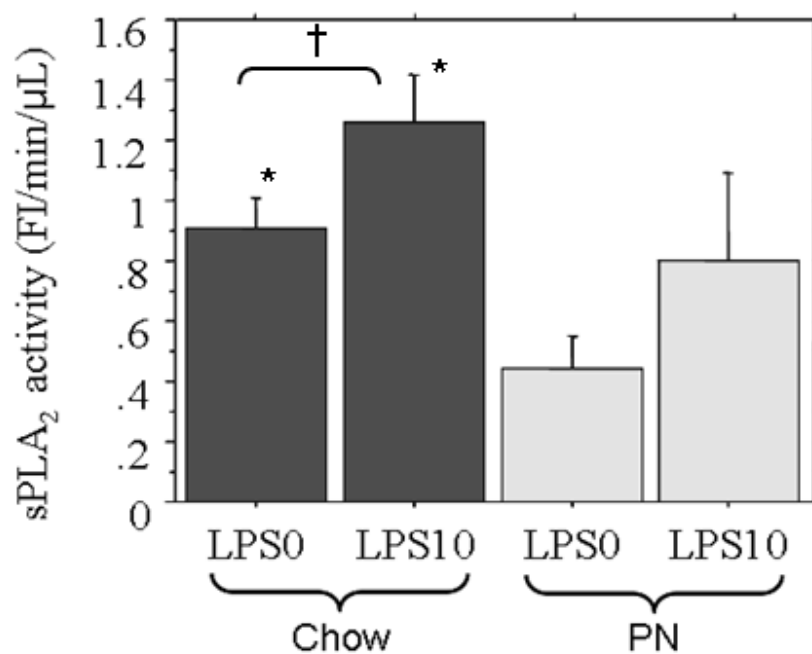
sPLA2 Activity of Chow or PN tissue secretions ± LPS stimulation. Following LPS stimulation (10 µg/mL LPS) chow significantly increased tissue culture sPLA2 activity compared with baseline (LPS0) and PN was increased but failed to reach significance due to variation in response. Both Chow LPS0 and LPS10 secretions were significantly greater than PN + LPS0. * $p<0.05$ vs PN+LPS0, † $p=0.02$.

Figure 4B.

14 kDa

Chow Chow PN PN
-LPS0 -LPS10 -LPS0 -LPS10
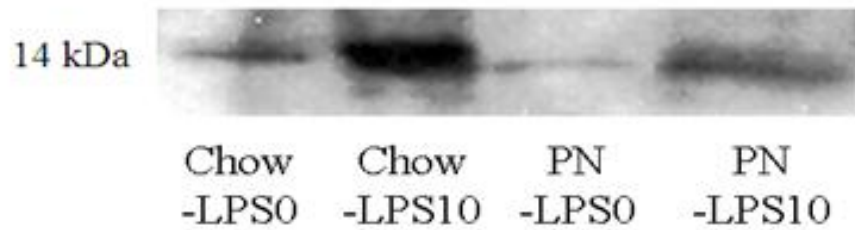
Representative western blot of sPLA2-IIA in tissue culture media from Chow or PN tissue showed LPS10 increased sPLA-IIA concentrations consistent with the quantified increase in sPLA2 acitivty observed in both groups. Bands are 14 kDa molecular weight.

Figure 5.
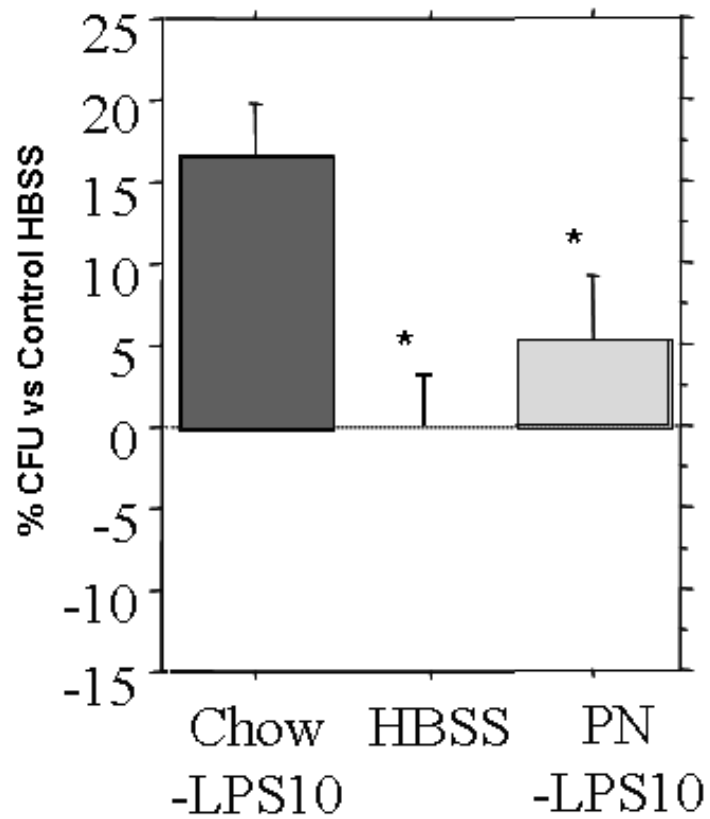
Bactericidal Activity of LPS stimulated secretions from Chow and PN. Bactericidal activity of Chow or PN tissue secretions following LPS (10 µg/mL LPS) stimulation. Chow had significantly greater bactericidal activity than HBSS control or PN. PN stimulated a sub-significant level of bactericidal activity. * $p<0.05$ vs Chow + LPS10

Figure 6.
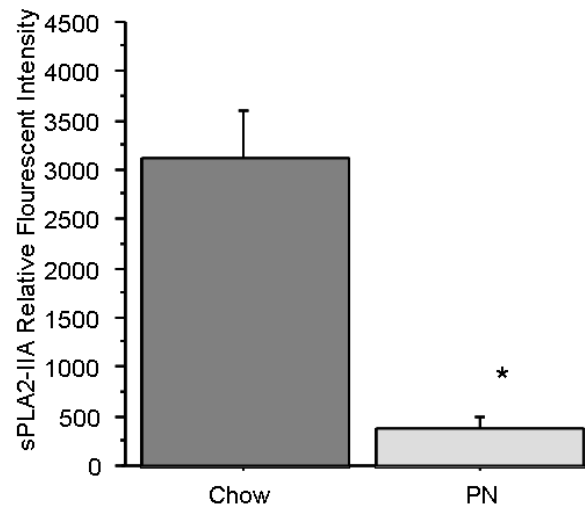
Relative Density of sPLA2 in Chow vs PN Ileum Tissue. Immunohistochemical analysis of sPLA2-IIA, performed by quantifying relative intensity of Fluorescent signal, showed Chow had significantly higher expression of sPLA2 compared with PN. * p<0.05 vs Chow

Figure 7.
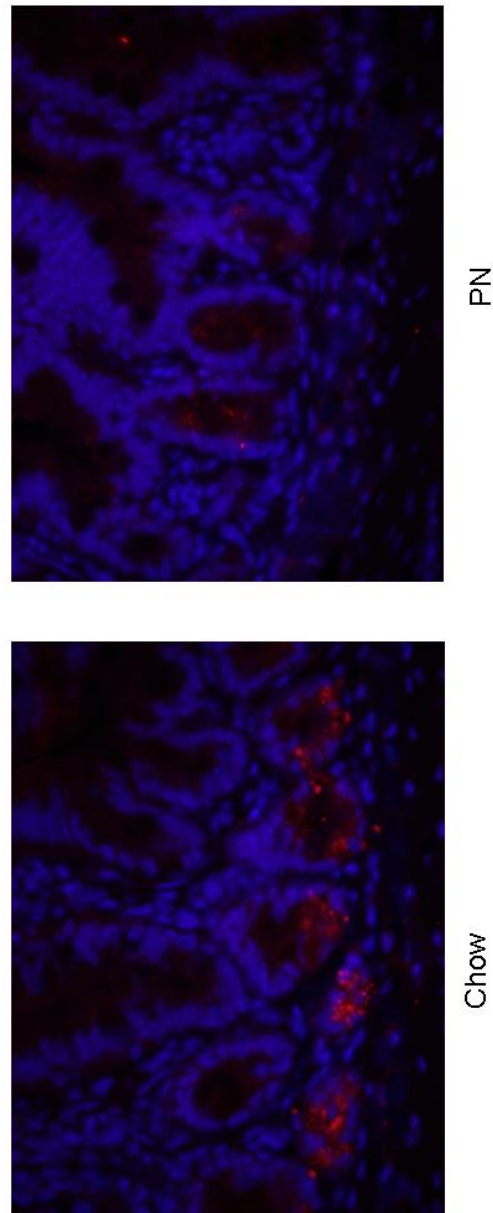
Immunohistochemistry of sPLA2 in Chow and PN ileum tissue
sPLA2-IIA (red) was localized in Paneth cell granules and was assessed in ileum samples of Chow and PN. Cell nuclei are stained with DAPI (blue).

Chapter 4

Cranberry Proanthocyanidins Improve the Gut Mucous Layer Morphology

and Function in Mice Receiving Elemental Enteral Nutrition.[1, 2, 8]

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ABSTRACT

Background: Lamina propria Th2 cytokines, interleukin (IL)–4 and IL-13, stimulate goblet cell (GC) proliferation and MUC2 production, which protect the intestinal mucosa. Elemental enteral nutrition (EEN) reduces tissue IL-4 and impairs barrier function. Proanthocyanidins (PACs) stimulate oral mucin levels. We hypothesized that adding PAC to EEN would maintain Th2— without stimulating Th1— cytokines and preserve luminal MUC2 vs EEN alone.

Materials and Methods: Seventy mice were randomized to 5 diet groups—standard chow, intragastric EEN, or EEN with lowPAC, midPAC (50 mg), or highPAC (100 mg PAC/kg BW) — for 5 days, starting 2 days after gastric cannulation. Ileal tissue was analyzed for histomorphology and the cytokines IL-4, IL-13, IL-1β, IL-6, and TNF–α by enzyme-linked immunosorbent assay. MUC2 was measured in intestinal washes.

Results: EEN lowered IL-13 ($P < .05$) compared with standard chow, whereas IL-4 was not significant ($P < .07$). LowPAC and midPAC increased IL-13 ($P < .05$), whereas highPAC increased both IL-4 and IL-13 ($P < .05$) compared with EEN. All EEN diets reduced ($P < .05$) crypt depth compared with the chow group. Compared with standard chow, GC numbers and luminal MUC2 were reduced with EEN ($P < .05$). These effects were attenuated ($P < .05$) with midPAC and highPAC. No changes were observed in tissue Th1 cytokines.

Conclusions: Adding PACs to EEN reverses impaired intestinal barrier function following EEN by improving the gut mucous layer and function through increased GC size and number as well as levels of MUC2 and ileal IL-4 and IL-13.

CLINICAL RELEVANCY

Multiple components of the intestinal mucosal barrier including secreted mucus and antimicrobial compounds maintain the host-bacterial relationship within the gut lumen. Elemental enteral nutrition adversely affects mucus production and secretion impairing the most basic level of gut immunity – barrier function. The addition of a complex, unabsorbed phytochemical, proanthocyanidins, to elemental nutrition improves this aspect of mucosal defense.

INTRODUCTION

Elemental enteral nutrition (EEN) is a therapeutic option for inflammatory bowel disorders such as Crohns disease [1]. Unfortunately, EEN induces well-defined dysfunction of the mucosal immune system, specifically within the gut-associated lymphoid tissue (GALT), and suppresses mucosal barrier function when compared to normal nutrition [2-5]. The integrity of the mucosal barrier is critical for maintaining the physical and chemical barrier against food and environmental antigens, including microbes [6]. The mucosal barrier is partly dependent upon the physical and compositional characteristics of the mucous layer [7]. Dietary compounds that affect this layer may have implications in health through modulation of the intestinal barrier [8].

PAC are a class of polyphenolic compounds widely distributed in plant-derived foods and beverages [9-11] that are associated with the prevention of chronic diseases in epidemiological studies [12-14]. However, PAC are minimally absorbed due to non-hydrolyzable bonds between monomeric subunits and a propensity to bind proteins through hydrogen bonding [15]. PAC

complex salivary glycoproteins, a process that causes astringency in the oral cavity when many fruits and beverages are ingested [16]. Complexation induces salivary excretion, hypertrophy of the parotid gland, and a shift in salivary composition to proline-rich glycoproteins in rodents [16, 17]. Because of poor absorption, greater than 95% of PAC remain in the intestinal lumen during transit [18, 19] suggesting beneficial dietary effects of PACs may occur through interactions at the mucosal surface of the gastrointestinal tract [8], for example, by influencing secretion of mucins, a class of glycoproteins, in the small intestine [14, 20].

Mucins are secreted by goblet cells (GC) and play a critical role in maintaining mucosal integrity [7]. GC, specialized intestinal epithelial cells, migrate up the villi after differentiating from crypt stem cells, turning over with the epithelial layer every 3-5 days. Mucin2 (MUC2) is the most abundant mucin secreted by intestinal GC. The importance of MUC2 is underscored in MUC2$^{-/-}$ mice, in which the deficiency leads to the development of lethal colitis [21]. MUC2 secretion is induced by cholinergic stimulation [22], while its production is regulated by the T-helper 2 (Th-2) cytokines IL-4 and IL-13, derived from lamina propria or intraepithelial lymphocytes [23-25].

In this study, we hypothesized that the addition of physiologically relevant doses [26, 27] of cranberry PAC (8-100 mg Gaelic Acid Equivalents (GAE) / kg body weight) to EEN would attenuate the negative effects of EEN on intestinal barrier function as determined by changes in the Th-2 cytokines IL-4 and IL-13, GC number and size, and luminal MUC2. Additionally, we examined potential changes in pro-inflammatory Th1 cytokines (IL-1β, IL-6, and TNF-α) [28, 29] and histomorphometric parameters (e.g., villi length and crypt depth) [30].

MATERIALS AND METHODS

PAC Preparation and Characterization

The methodology for PAC preparation and characterization was previously published [31]. Briefly, Non-depectinized cranberry presscake was ground with liquid nitrogen and extracted with 70% acetone (Fisher Scientific, Fair Lawn, NJ). Samples were sonicated and centrifuged at for 10 minutes. The extraction was repeated twice. Acetone was removed by evaporation and the aqueous suspension was solubilized in ethanol (Decon Labs Inc., King of Prussia, PA), followed by centrifugation to eliminate ethanol insoluble material. Cranberry presscake crude extract was loaded on a Sephadex LH-20™ (GE Healthcare, Uppsala, Sweden) column and PAC were isolated by sequential elution with ethanol, ethanol/methanol (1:1) and 80% acetone. Acetone in the last fraction that contained PAC was removed by evaporation under vacuum and re-solubilized in methanol (Fisher Scientific, Fair Lawn, NJ). The total phenolic content of the PAC fraction was determined by the modified Folin-Ciocalteu method and reported as gallic acid equivalents (GAE).

An aliquot of the cranberry presscake PAC fraction was diluted tenfold and a sample was injected onto a Waters Spherisorb® 10 µm ODS2 RP-18 column. The solvents for elution were trifluoroacetic acid/water (0.1%) and methanol. The HPLC system consisted of a Waters automated gradient controller, two Waters 501 HPLC pumps, and a Rheodyne 7125 manual injector. The elution was monitored by a Waters 996 diode array detector using Waters Millennium software for collecting and analyzing three-dimensional chromatograms.

An aliquot of the cranberry presscake PAC fraction was mixed with 2,5-dihydroxybenzoic acid (Aldrich, Milwaukee, WI) and the mixture was applied onto a MALDI-TOF MS stainless steel target and dried at room temperature. Mass spectra were collected on a Bruker Reflex II MALDI-TOF-MS (Billerica, MA) equipped with delayed extraction and a N_2 laser (337 nm) in order to characterize the range in degree of polymerization (DP) and nature of interflavan bonds in the cranberry PAC. All preparations were analyzed in the positive ion linear and reflectron mode to detect $[M+Na]^+$ and $[M+K]^+$ molecular ions. MALDI-TOF MS is ideally suited for characterizing PAC because, unlike electrospray ionization in which multiple charge molecular ions create very complex spectral peaks that are often difficult to interpret, this mass spectral technique produces only a singly charged molecular ion for each parent molecule [11].

Animals

All animal experiment protocols were approved by Animal Care and Use Committee of the University of Wisconsin-Madison and the Middleton Veterans Administration Hospital, Madison. Male Institute of Cancer Research (ICR) outbred mice were purchased through Harlan (Indianapolis, IN) and housed in an American Association for Accreditation of Laboratory Animal Care-accredited conventional facility on the V.A Williamson Hospital Campus. The mice were acclimatized for one week in a temperature and humidity controlled environment with a 12h/12h light/dark cycle. The mice were housed 5 per micro isolater-top cages and fed *ad libitum* Chow (Rodent Diet 5001, LabDiet, PMI Nutrition International, St. Louis, MO) and water for 1 week prior to initiation of study protocol. A description and detailed chemical

composition of Rodent Diet 5001 is available at http://labdiet.com/pdf/5001.pdf. Once entering

study protocol, the mice were housed individually in metal wire-bottomed cages to prevent

coprophagia and ingestion of bedding.

Experimental Design

Seventy male ICR mice (6 to 8 wk old) were randomized by weight (n = 14 / diet group)

to receive Standard Chow, intragastric EEN or intragastric EEN+PAC [8 mg (EEN+lowPAC),

50 mg (EEN+midPAC) or 100 mg (EEN+highPAC) GAE of PAC/kg body weight]. Animals

were anesthetized with intraperitoneal administration of ketamine (100 mg/kg) and acepromazine

(10 mg/kg) and gastrostomy was performed. Catheters were tunneled subcutaneously from the

gastrostomy site, over the back, finally exiting mid-tail. The mice were partially restrained by the

tail for the remainder of the study to protect the catheter during infusions. This partial restraint

technique does not induce significant stress in the mice [32]. The catheterized mice were connected

to infusion pumps and allowed to recover for 48 h while receiving 4 mL/d of saline (0.9%) via

the catheter. The mice also received *ad libitum* Chow (Rodent Diet 5001, LabDiet) and water.

Following the recovery period, animals received their assigned dietary treatments. The

Standard Chow fed mice were given *ad libitum* chow diet and water, and continued to receive

0.9% saline at 4 mL/d via the intragastric catheter. EEN and EEN+PAC fed mice received

solution at 4 mL/d (day 1), 7 mL/d (day 2) and 10 mL/d (days 3-5) as well as *ad libitum* water

throughout the study. The EEN solution includes 6.0% amino acids, 35.6% dextrose, electrolytes,

and multivitamins, with a non-protein calorie to nitrogen ratio of 126.1 (527.0 kJ/g nitrogen).

This value meets the calculated nutrient requirements of mice weighing 30 to 35 g [33].

After 5 d of feeding (7 days post-catheterization), mice were weighed, anesthetized as before, and exsanguinated via left axillary artery transection. The small intestine from each mouse was removed and the lumen rinsed with 20 mL HBSS (Bio Whittaker, Walkersville, MD). The luminal rinse was centrifuged at 2,000 x g for 10 min, and supernatant was aliquoted and frozen at -80ºC for MUC2 analysis. Ileal tissue samples were obtained from a 3 cm segment of ileum that excluded Peyer's patches. Samples for cytokine determination were flash-frozen in liquid N_2 with 1% protease inhibitor cocktail (p8340, Sigma-Aldrich, St. Louis, MO) and stored at -80ºC until subsequent analysis, while samples for GC analysis were fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, and stored at 4ºC until subsequent histology.

Analysis of ileal cytokines

The flash-frozen small intestine segment from each animal was homogenized in RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1% protease inhibitor cocktail (Sigma-Aldrich). The homogenate was kept on ice for 30 min prior to centrifugation at 16,000 x g for 10 min at 4ºC. The supernatant was then stored at –20ºC until analysis. Prior to storage, the protein concentration of the supernatant was determined by the Bradford method using BSA as a standard.

Concentrations of IL-4, IL-13, IL-1β, IL-6, and TNF-α were determined in the supernatant using solid phase sandwich ELISA kits (BD Biosciences, San Diego, CA), according to manufacturer's instructions and identical to our previous work [5, 34]. The absorbance at 450 nm was determined using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The respective cytokine concentrations in the samples were determined by using a 4-parameter logistic fit standard curve (SOFTmax PRO software; Molecular Devices; Sunnyvale, CA) and normalized to total tissue protein content.

Analysis of luminal MUC2

Our method of MUC2 analysis was similar to previous work [35-38]. Proteins in the intestinal wash fluid (4μL) from each animal were separated by 10% agarose gel by electrophoresis at 150V for 80 min at room temperature. The resolved proteins were transferred to polyvinylidene fluoride membrane using tris-glycine buffer containing 20% methanol at 80V for 60 min at 4ºC. The membrane was blocked with 5% nonfat dry milk prepared in Tris buffered saline containing Tween (0.05%) for 1 h at room temperature with constant agitation. Then, the membrane was incubated with mouse anti-human MUC2 (ab-11197, Abcam Inc, Cambridge, MA) primary antibody (diluted 1:2500) overnight at 4ºC with constant agitation. The membrane was washed and incubated with stabilized goat anti-mouse IgG-HRP conjugate (sc-2005, Santa Cruz Biotechnology, CA) secondary antibody (diluted 1:20,000) for 1 h at room temperature with constant agitation. After washing, the membrane was incubated with HRP

substrate (Super Signal West Femto substrate; Pierce, Rockford, IL) for 5 min and the protein of interest (MUC2) was detected using photographic film. The relative intensities of both the monomeric and dimeric forms of MUC2 were determined together for each sample using NIH ImageJ software (version 1.43, http://rsbweb.nih.gov/ij/); internal controls were used to normalize the densitometry across multiple films.

Histomorphometric analysis

The fixed ileal tissue sections were processed (Tissue-Tek V.I.P, Sakura Finetek, Torrance, CA), and embedded in paraffin. The embedded tissue was cut (5 µm thick), deparaffinized, rehydrated through graded ethanol washes (100% ethanol x 2, 95% ethanol x 2, 70% ethanol x 1, 2 min each) and placed into distilled H_2O. Samples were stained with periodic acid-schiff (PAS) and counterstained with hematoxylin. GC number was determined by determining the average number of GC present in 15 individual villi per animal. GC size (μm^2) was obtained by imaging tissue sections and analyzing individual GC area with NIH ImageJ software (version 1.43, http://rsbweb.nih.gov/ij/). Villi length and crypt depth measurements were determined in 15 villi and crypts. The histomorphometric measurements were performed by two independent, blinded researchers.

Statistical analysis

A fixed effects ANOVA model was fit for each measured parameter using the PROC MIXED function of the statistical software (SAS Software (Version 8), SAS Institute Inc, Cary, NC) to test for significant effects of diet. The correlations between observations between diet groups were modeled using a diagonal covariance structure. For each measured parameter, the model was fit using the untransformed data, and the residuals were evaluated to ensure that standard ANOVA assumptions of constant variance and normality were reasonably met. Transformations of the data were performed if required to improve adherence to these assumptions. Type III tests were then performed to evaluate the significance of the effects of interest for each measured parameter, and least-square means were calculated for the diet groups. Primary effects of interest were differences between the: (1) Standard Chow and EEN groups, (2) EEN and EEN+PAC groups (at each dose), and (3) Standard Chow and EEN+PAC groups (at each dose). The Standard Chow group was included in analysis as a positive control as done in all of our previous work. The data are reported as least-square mean ± standard error of mean (SEM). Statistical significance was accepted at $p < 0.05$.

RESULTS

PAC characterization by HPLC and MALDI-TOF MS

The cranberry presscake PAC eluted as two unresolved peaks that had absorbance at 280 nm and minor absorbance at 520 nm due to the presence of covalently linked anthocyanin-proanthocyanidin pigments. No peaks were observed with an absorbance max typical of the other classes of cranberry polyphenolic compounds (anthocyanins, hydroxycinnamic acids, and

flavonols). The poorly resolved chromatogram at 280 nm is due to structural heterogeneity of cranberry presscake PAC [11].

Reflectron mode MALDI-TOF MS showed masses that correspond to PAC with at least 1A-type interflavan bond in trimers to undecamers. MALDI-TOF MS linear mode spectra had m/z peaks that correspond to cranberry presscake PAC with a range of 3 to 23 degrees of polymerization. The spectra also contained m/z peaks that correspond to covalently linked anthocyanin-proanthocyanidin molecules, ranging from monomers to heptamers (data not shown).

Body Weight Changes

Pre-experiment body weights did not significantly differ between treatment groups. Post-experiment body weights were significantly ($p < 0.05$) lower in all EEN fed groups compared with standard Chow [Table 2]. The decrease in body weight observed in EEN groups is partly due to absence of bowel fecal content, which we have measured previously at 1-1.5 grams. Post-experiment body weight between EEN fed groups did not differ.

Analysis of ileal cytokines

IL-4 level in the ileal tissue of the EEN group was lower than in the Standard Chow group, almost reaching statistical significance (P = 0.051) [Table 3]. IL-4 levels in the EEN+highPAC group was significantly higher than in the EEN group (P < 0.005), while levels in EEN+lowPAC nor EEN+midPAC groups significantly differed from the EEN group. Additionally, Tissue IL-4 was significantly greater in EEN+highPAC than EEN+lowPAC (P < 0.005).

EEN significantly reduced IL-13 in the ileal tissue compared to Standard Chow (P < 0.05). IL-13 levels in the EEN+lowPAC (P< 0.05), EEN+midPAC (P < 0.05), and EEN+highPAC (P < 0.005) were significantly higher than in the EEN group alone.

Compared with Standard Chow, EEN did not significantly affect the Th1 cytokines, IL-1β, IL-6, or TNF-α; the addition of PACs at any dose had no effect on these cytokines.

Analysis of GC number and size

While the length of villi were decreased in all EEN fed groups compared with Standard Chow, these changes were not significant. However, there was a significant reduction in crypt depth with all EEN diets (P< 0.05) compared with Standard Chow. The addition of PAC to EEN had no significant effect upon villi length or crypt depth compared with the EEN alone [Table 4].

EEN significantly reduced the number of GCs per villi compared with Standard Chow (P < 0.005). EEN+lowPAC (P < 0.05), EEN+midPAC (P < 0.01), and EEN+highPAC (P < 0.0001) significantly increased the number of GCs per villi compared with EEN alone. The

number of GCs per villi in the EEN+highPAC was significantly greater than the EEN+lowPAC group ($P < 0.05$). When adjusted for villi length (GCs/µL villi length) in EEN, there were no significant differences between EEN and Standard Chow in the number of GCs ($P = 0.12$). However, there were more GCs/villi length in the EEN+midPAC ($P = 0.05$) and EEN+highPAC ($P<0.01$) compared with EEN alone. A representative histomorphometric image is shown for Standard Chow, EEN, and EEN+highPAC [Figure 1].

Although the GC size (μm^2) in the EEN group was smaller than in the Standard Chow group, this difference was not significant ($P = 0.29$) [Table 4]. The GC sizes in the EEN+lowPAC ($P < 0.05$), EEN+midPAC ($P < 0.01$), and EEN+highPAC ($P < 0.05$) groups were significantly greater than EEN alone.

Analysis of luminal MUC2

The monomer and dimer observed, at molecular weight markers 250 and 500 kDa respectively, were consistent with other reports of the highly oligomeric structure of intestinal MUC2 [35-38]. The relative luminal MUC2 [Figure 2] in the EEN and EEN+lowPAC groups was lower than the Standard Chow group, although these differences failed to reach significance ($P = 0.057$). However, the relative luminal MUC2 in the EEN+highPAC ($P <0.005$) group was higher than EEN alone, but the EEN+midPAC ($P = 0.06$) group failed to reach significance. Additionally, the level of MUC2 in the EEN+highPAC was significantly greater than EEN+lowPAC ($P < 0.05$).

DISCUSSION

This study demonstrates that the addition of cranberry PAC to EEN solution improves ileal tissue IL-4 and IL-13 levels, GC number and size, and the secretion of intestinal MUC2, which likely contribute to the impairment of the mucosal barrier integrity previously observed by EEN alone [2, 39]. The gastrointestinal mucosa maintains a physical and chemical barrier against 100 trillion resident bacteria as well as food and environmental antigens [6]. A number of interrelated factors influence this function, including mucus glycoproteins, antimicrobial molecules, specific and non-specific antibodies, enterocyte tight-junctions, and colonization of a commensal microbiota [40, 41]. Dietary intake of the host affects the complex interplay between these factors [42, 43]. The route and complexity of nutrition profoundly influences the mucosal immune system, specifically the mucosal associated lymphoid tissue [4, 5, 44]. A reduction in dietary intake or complexity, such as those that occur with parenteral nutrition or administration of EEN, decreases the number of lymphocytes in Peyer's patches and lamina propria, reduces levels of IgA-stimulating Th-2 type cytokines in the gut wall, and reduces levels of intestinal immunoglobulins (primarily IgA) compared to the feeding of a Standard Chow diet or administration of a complex enteral diet containing complex carbohydrates, proteins and fats [4, 34, 44, 45]. EEN also increases barrier permeability and significantly suppresses bacterial diversity within the gut [2, 39]. While the influence of dietary intake or complexity on mucosal barrier and immunity is appreciated [41], very little is known of the influence of "non-nutritive" dietary compounds such as PAC.

PAC are complex oligomeric polyphenolic compounds widely distributed in fruits, including grapes, cranberries, and apples, and other foods and beverages such as chocolate and

wine [9-11]. Epidemiological studies suggest PAC may have a wide range of beneficial health effects [12-14]. However, PAC are minimally absorbed across the enterocyte layer due to non-hydrolyzable bonds between flavan-3-ol monomeric units and their ability to complex both dietary and endogenous proteins [15]. Further, PAC oligomers range in DP from 3 to 30, or more, and therefore have higher molecular weight than other common plant polyphenols. Consequentially, greater than 95% of PAC remain in the intestinal lumen during transit through the gastrointestinal tract [46-48].

Since PAC are poorly absorbed, a number of mechanisms have been investigated to explain their potential beneficial effects. PAC have been shown to exert antioxidant and non-specific antimicrobial functions within the gut [20]. Recent animal studies also demonstrate the addition of dietary PAC palliates chemically-induced colitis, although the mechanism of this remains unclear [49-51]. Another important effect of PAC is their propensity to complex salivary glycoproteins when ingested, a process that causes the astringency of many fruits and beverages [10]. Astringency occurs when PAC crosslink and precipitate salivary glycoproteins and PAC with higher DP have greater effects on crosslinking and precipitation [52]. Several biological effects occur in response to astringency including increased salivary excretion, hypertrophy of the parotid gland, and shift in salivary composition to proline rich proteins [16]. Within the intestine, *in vitro* studies demonstrate that intra-epithelial γδ T lymphocytes, in response to PAC, activate and proliferate [53]. Interestingly, the level of γδ T cell response also increases with greater DP of PAC. These observations not only suggest that PAC may play an influential role in context of mucosal barrier physiology and immunity, but that DP of PAC may be of importance when investigating their effects. Accordingly, we previously characterized the PAC used in this experiment [31]. This

analysis allows for the characterization and reliable reproduction of chromatographic fractions for inclusion in experimental treatments.

In this study, we investigated the effects of addition of cranberry PAC to EEN solution on ileal tissue cytokine levels, morphology including GC number and size, and the secretion of the primary glycoprotein MUC2, and explored the effect of physiological doses of PAC on these parameters. We used a chemically defined EEN solution administered via a gastrostomy tube as a model of an elemental enteral diet that we have previously utilized. The EEN administration results in reproducible effects on intestinal (and respiratory) mucosal immunity allowing examination of changes induced with PAC.

Compared to Chow, EEN produces significantly fewer total GCs per villi. However, when normalizing the GC numbers over villi length (GC number/*µm)*, there were no differences between EEN and the Standard Chow diet. There were also no significant differences in GC size and villi length between Standard Chow and EEN, although EEN reduced the average measurement of both parameters. Interestingly, the number of GCs per villi length was significantly preserved in the EEN+midPAC and EEN+highPAC groups compared to EEN alone. GCs normally undergo hypertrophy and hyperplasia in response to IL-4 and IL-13, which act through the IL-4 receptor α and IL-13 receptor α1, respectively [23, 54, 55]. Our data shows that EEN lowered ileal IL-4 and IL-13 levels compared with Standard Chow. The addition of PAC to the EEN diet maintained IL-4 and IL-13, but did not significantly affect the cytokines IL-1β, TNF-α, or IL-6 [Table 3]. Since GC differentiate, migrate up the villi, and slough off every 3-5 days, these findings suggests the addition of PAC to the EEN diet alters the rate of cellular differentiation of progenitor crypt stem cells to GC likely via changes in Th-2 type cytokines

observed [4]. The data also suggest PAC induce the observed effect through Th2 mediated immunity consistent with a previous study showing a similar IL-4 effect in colonic tissue following ingestion of proanthocyanidins [50]. Additionally, while the effects on the Th1 cytokine TNF-α were not significant across treatment groups, the trend of reduced tissue TNF-α level with increasing doses of PAC was consistent with previous work [56].

Simultaneously, EEN suppressed the concentration of MUC2 within the lumen, although this change did not reach significance. Functionally MUC2 forms the viscous mucin layer which overlays the intestinal surface, allowing smooth passage of digesta. From an immunological stand point, secreted antimicrobial proteins and peptides from Paneth cells as well as secretory IgA (sIgA) localize and are concentrated in this layer [57]. These mucin glycoproteins also provide endogenous flora with a consistent nutrient source. The observed decrease in luminal MUC2 may increase susceptibility to bacterial opportunistic pathogens or intestinal inflammation, since others have shown that MUC2$^{-/-}$ mice are at increased risk for spontaneous colitis [21]. The addition of PAC at the EEN+midPAC and EEN+highPAC doses maintained MUC2 to levels observed in the Standard Chow group.

Cranberry PAC administration at physiologic doses [26, 27] counteracts many of the changes associated with EEN administration. One limitation of the current study is that we do not address the source of the Th2 cytokines, although studies investigating changes to tissue lymphocytes and whether a mechanism similar to astringency is responsible for these observations effects are planned. Overall, this study supports the hypothesis that reduced enteral stimulation results in the impairment of mucosal integrity and gut barrier function through the reduction in the mucin component. The current work demonstrates that the administration of EEN produces lower levels

of the Th2 stimulating cytokine IL-13, lower GC number and size, and lower luminal MUC2 levels in the ileum. The addition of cranberry PAC to this diet, at physiologic doses, attenuates these changes and likely normalizes mucosal integrity. This suggests that a non-nutritional dietary component such as PAC may influence health without being absorbed from the gastrointestinal tract.

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Table 1: Formulation of EEN Solution

Component	Amount (per 1 L)
Dextrose	356.0 g
Amino acids (Clinisol)	60.0 g
Sodium chloride	32.0 mEq
Sodium phosphate	36 mmol
Potassium chloride	16 mEq
Calcium gluconate	37.5 mEq
Potassium acetate	44.0 mEq
Magnesium sulfate	8.0 mEq
Manganese	0.8 mg
Copper	0.5 μg
Zinc	2.0 mg
Vitamin C	200 mg
Vitamin A	3300 IU
Vitamin D3	200 IU
Thiamine	6 mg
Riboflavan	3.6 mg
Pyridoxine HCl	6 mg
Niacinamide	40 mg
Folic Acid	600 mcg
Biotin	60 mcg
Cyanocobalamin	5 mcg
Vitamin E (*dl*-α-tocopheryl Acetate)	10 IU
Vitamin K1	150 mcg
Dexpanthenol	15 mg

Table 2: Experimental Body Weights[1].

	Chow	EEN	EEN + lowPAC	EEN + midPAC	EEN + highPAC
Pre-Experiment Weight (grams)	32.88 ± 0.53	34.23 ± 0.60	32.44 ± 0.85	34.14 ± 0.73	33.40 ± 0.60
Post-Experiment Weight (grams)	30.44 ± 0.87	27.29 ± 0.67*	26.71 ± 0.62*	28.02 ± 0.76*	27.64 ± 0.52*

[1] Values are mean ± SEM, n = 12-14. * denotes $P < 0.05$ vs Chow. N/S, non-significant effect across groups.

EEN Elemental Enteral Diet.; EEN+lowPAC, EEN with 8 mg/kg BW PAC; EEN+midPAC, EEN with 50 mg/kg BW PAC; EEN+highPAC, EEN with 100 mg/kg BW.

Table 3: Effects of Feeding Chow, EEN, EEN+lowPAC, EEN+midPAC, and EEN+highPAC Diets on Intestinal Tissue Cytokines, IL-4, IL-13, IL-1β, IL-6, and TNF-α[1].

pg/mg Protein	Chow	EEN	EEN + lowPAC	EEN + midPAC	EEN + highPAC
IL-4	6.01 ± 0.56	4.48 ± 0.52	4.98 ± 0.52	5.81 ± 0.52	$6.99\ 0.52$ ‡
IL-13	11.37 ± 1.63	7.54 ± 1.42 *	10.94 ± 1.42 ‡	11.83 ± 1.42 ‡	13.94 ± 1.79 ‡
IL-6	7.55 ± 0.78 N/S	6.94 ± 0.75	7.19 ± 0.75	7.40 ± 0.75	7.04 ± 0.73
TNF-α	14.74 ± 2.67 N/S	18.29 ± 2.38	17.63 ± 2.46	17.69 ± 2.38	12.83 ± 2.38
IL-1β	167.7 ± 18.06 N/S	153.7 ± 16.8	129.3 ± 18.8	166.8 ± 16.8	114.7 ± 16.8

[1] Values are mean ± SEM, n = 6–14. * denotes $P < 0.05$ vs Chow. ‡ denotes $P < 0.05$ vs. EEN. N/S, non-significant effect across groups.

EEN Elemental Enteral Diet.; EEN+lowPAC, EEN with 8 mg/kg BW PAC; EEN+midPAC, EEN with 50 mg/kg BW PAC; EEN+highPAC, EEN with 100 mg/kg BW.

Table 4: Effects of Feeding Chow, EEN, EEN+lowPAC, EEN+midPAC, and EEN+highPAC on Intestinal Histomorphometry[1].

Intestinal Parameter	Chow	EEN	EEN + lowPAC	EEN + midPAC	EEN + highPAC
Villi Length, μm	171.5 ± 5.49 N/S	155.5 ± 5.14	152.5 ± 5.50	157.0 ± 5.50	155.4 ± 5.50
Crypt Depth, μm	79.99 ± 2.73	64.83 ± 2.55 *	66.14 ± 2.73 *	64.17 ± 2.73 *	66.17 ± 2.73 *
GC/Villi, N	9.72 ± 0.44	7.98 ± 0.36	9.15 ± 0.44 ‡	9.66 ± 0.48 ‡	10.37 ± 0.39 ‡,†
GC/Villi Length, N/μm	0.0598 ± 0.0036	0.0522 ± 0.0033	0.0598 ± 0.0036	0.0619 ± 0.0036 ‡	0.0657 ± 0.0036 ‡
GC area, μm^2	54.49	48.56	62.57 ‡	64.50 ‡	61.50 ‡

[1] Values are mean ± SEM, n = 6–14. * denotes P < 0.05 vs Chow. ‡ denotes P < 0.05 vs. EEN. N/S, non-significant effect across groups.

EEN Elemental Enteral Diet.; EEN+lowPAC, EEN with 8 mg/kg BW PAC; EEN+midPAC, EEN with 50 mg/kg BW PAC; EEN+highPAC, EEN with 100 mg/kg BW.

Figure 1
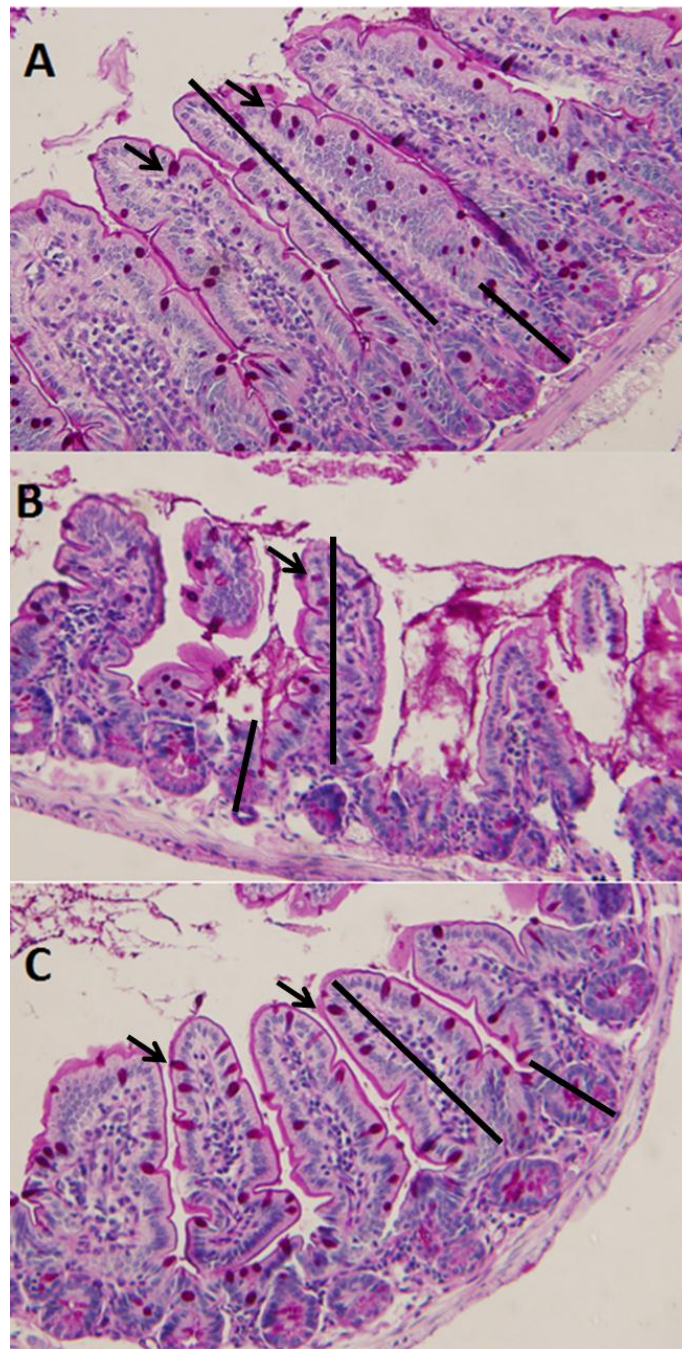
Representative Image of Periodic Acid Shiff-Base (PAS) Stained Ileum Tissue from (A) Chow, (B) EEN, and (C) EEN+highPAC. Goblet cells are stained pink (denoted by arrows). Measurements of villi length and crypt depth were made as indicated. 20x Zoom.

Figure 2
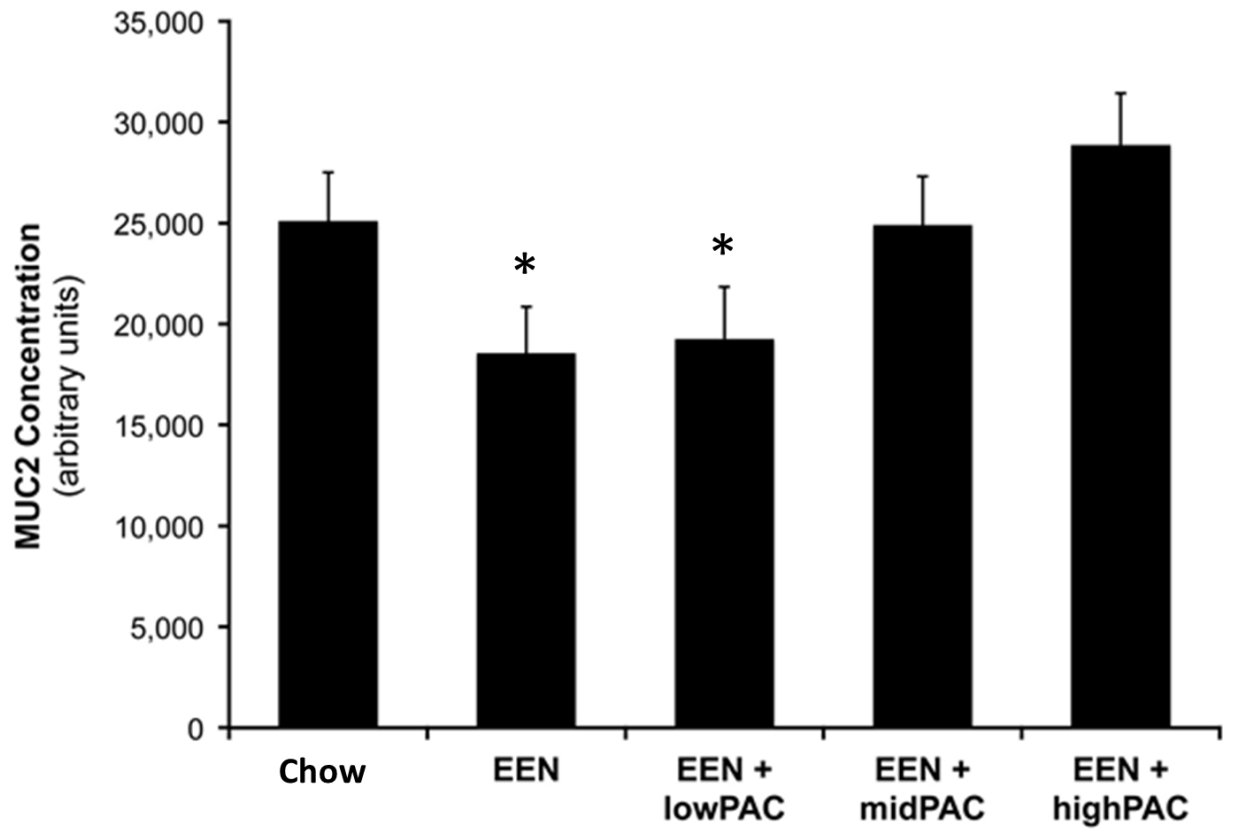
Effects of Feeding Chow, EEN, EEN+lowPAC, EEN+midPAC, and EEN+highPAC Diets on Intestinal Lumen MUC2 Displayed in Arbitrary Units. * denotes $P < 0.05$ vs EEN+highPAC.

Chapter 5

Cranberry Proanthocyanidins Maintain Intestinal sIgA During

Elemental Enteral Nutrition

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Under Peer Review

ABSTRACT

Scope: Elemental enteral nutrition (EEN) decreases gut-associated lymphoid tissue (GALT) function, including fewer Peyer's patch lymphocytes, lower levels of the tissue Th2 cytokines and mucosal transport protein polymeric immunoglobulin receptor (pIgR), leading to lower luminal sIgA levels. Since we recently demonstrated cranberry proanthocyanidins (PACs) maintain the Th2 cytokine IL-4 when added to EEN, we hypothesized the addition of PACs to EEN would normalize other GALT parameters and maintain luminal levels of sIgA.

Methods and Results: ICR mice were randomized (12/group) to receive Chow, EEN, or EEN+PACs (100 mg/kg body weight) for 5 days, starting 2 days after intra-gastric cannulation. Ileum tissue was collected to measure IL-4 by ELISA, pIgR by western blot, and phosphorylated STAT6 by microarray. Intestinal wash fluid was collected to measure sIgA by western blot. Compared with Chow, EEN significantly decreased tissue IL-4, Phosphorylated STAT6, and pIgR. The addition of PACs to EEN prevented these alterations. Compared with Chow, EEN resulted in significantly lower levels of luminal sIgA. The addition of PACs to EEN increased luminal sIgA levels compared to EEN alone.

Conclusions: This study suggests the addition of PACs to EEN may support GALT function and maintain intestinal sIgA levels compared with EEN alimentation alone.

INTRODUCTION

Elemental enteral nutrition (EEN) is a useful therapy option for conditions requiring a reduced residual diet, including inflammatory bowel diseases and pancreatitis [1-3]. Decreased dietary bulk and complexity provided with EEN attenuates mucosal agitation and painful symptoms. Unfortunately, reduced dietary complexity, such as provided with EEN or parenteral nutrition (PN), alters the structure and function of the gut-associated lymphoid tissue (GALT). Ultimately, reduced dietary complexity manifests as decreased secretory immunoglobulin-A (sIgA) in the gut lumen compared to enteral feeds [4-7]. sIgA is the primary protective compound of acquired immunity secreted by the host mucosa, which among other notable functions exclude enteric bacteria from attachment to the host [8, 9]. EEN also results in increased bacterial translocation and decreased microbiome diversity [9, 10]. To address EEN induced susceptibilities various interventions, including the use of natural products, have been investigated to provide anti-inflammatory and protective effects in the gut [7]. Since our established feeding model employing intra-gastric administered EEN results in the reproducible loss of intestinal (and respiratory) sIgA, this work investigated whether a class of natural compounds isolated from cranberries, proanthocyanidins (PACs), support mucosal protection by stimulating luminal sIgA levels when added to EEN.

PACs are a class of polyphenols that are found in many dietary sources, including fruits, tea, chocolate, and wine [11, 12] that epidemiological studies suggest may prevent the onset of chronic pathologies, such as cardiovascular disease and cancer [13, 14]. Interestingly, greater than 95% of ingested PACs remain in the gut lumen during transit, limiting their interaction with systemic compartments and making benefits of PACs ingestion upon health unique compared

with other polyphenolics. We recently investigated the effect of adding PACs to EEN in an

animal model and observed increased tissue Th2 cytokines levels, including IL-4 and IL-13 [15].

Increased IL-4 tissue levels have also been observed in experimental colitis following PACs

supplementation [16].

Our previous work demonstrates reduced luminal sIgA levels following EEN or

parenteral nutrition is multifactorial, including fewer lymphocyte numbers in both Peyer's

patches (PP) and lamina propria compartments; suppressed T helper 2 cytokines (Th2), IL-4 and

IL-10, in the lamina propria; and reduced expression of mucosal pIgR, which is the primary

transport protein for sIgA [17-20]. Expression of pIgR is regulated in part through the Janus

kinase/signal transducer and activator of transcription (JAK/STAT) pathway, a cytokine

signaling cascade used to transduce a wide array of cellular events [21-24]. IL-4 binds the IL-4

receptor-α inducing intracellular STAT6-phosphoyrlation, dimerization, and migration into the

cell nucleus targeting transcription products, including pIgR [25]. Since EEN decreases sIgA

levels and our recent work suggest PACs support intestinal Th2 cytokines, we hypothesized that

the addition of physiological PACs doses [12] to EEN would support GALT function and

luminal sIgA compared with EEN alimentation alone.

MATERIALS AND METHODS

PAC Preparation and Characterization

The PAC preparation and characterization for use in experimental diets for this study

were previously published [26]. Briefly, non-depectinized cranberry presscake was ground with

liquid nitrogen and extracted with 70% acetone three times (Fisher Scientific, Fair Lawn, NJ). Acetone was removed by evaporation and the aqueous suspension was solubilized in ethanol (Decon Labs Inc., King of Prussia, PA), followed by centrifugation to eliminate ethanol insoluble material. Cranberry presscake crude extract was loaded on a Sephadex LH-20™ (GE Healthcare, Uppsala, Sweden) column and PAC were isolated by sequential elution with ethanol, ethanol/methanol (1:1) and 80% acetone. Acetone in the last fraction that contained PAC was removed by evaporation under vacuum and re-solubilized in methanol (Fisher Scientific, Fair Lawn, NJ). The total phenolic content of the PAC fraction was determined by Folin-Ciocalteu method and reported as Gallic acid equivalents (GAE).

An aliquot of the cranberry presscake PAC fraction was diluted tenfold and a sample was injected onto a Waters Spherisorb® 10 μm ODS2 RP-18 column. The solvents for elution were trifluoroacetic acid/water (0.1%) and methanol. The HPLC system consisted of a Waters automated gradient controller, two Waters 501 HPLC pumps, and a Rheodyne 7125 manual injector. The elution was monitored by a Waters 996 diode array detector using Waters Millennium software for collecting and analyzing three-dimensional chromatograms.

An aliquot of the cranberry presscake PAC fraction was mixed with 2,5-dihydroxybenzoic acid (Aldrich, Milwaukee, WI) and the mixture was applied onto a MALDI-TOF MS stainless steel target and dried at room temperature. Mass spectra were collected on a Bruker Reflex II MALDI-TOF-MS (Billerica, MA) equipped with delayed extraction and a N_2 laser (337 nm) in order to characterize the range in degree of polymerization (DP) and nature of interflavan bonds in the cranberry PAC. All preparations were analyzed in the positive ion linear and reflectron mode to detect $[M+Na]^+$ and $[M+K]^+$ molecular ions. MALDI-TOF MS is ideally

suited for characterizing PAC because, unlike electrospray ionization in which multiple charge

molecular ions create very complex spectral peaks that are often difficult to interpret, this mass

spectral technique produces only a singly charged molecular ion for each parent molecule [27]

Animals

The Animal Care and Use Committee of the University of Wisconsin-Madison and

Middleton Veterans Administration Hospital, Madison approved all animal experimental

protocols. Male Institute of Cancer Research (ICR) mice were purchased through Harlan

(Indianapolis, IN) and housed in an American Association for Accreditation of Laboratory

Animal Care-accredited conventional facility on the V.A Williamson Hospital Campus. Mice

were acclimatized for one week in an environment controlled for temperature and humidity with

a 12/12-hour light/dark cycle. Mice were housed 5 per covered/filtered box and fed *ad libitum*

chow (LabDiet, PMI Nutrition International, St. Louis, MO) and water for 1 week prior to

initiation of study protocol. After entering study protocol mice were housed individually in

metal cages with wire grid floors to prevent coprophagia and bedding ingestion.

Experimental Design

Male ICR mice, ages 6 to 8 weeks, were randomized to Chow with a gastric catheter (n =

12), intragastric elemental nutrition (EEN) (n=12) via gastrostomy, or EEN + PACs via

gastrostomy (100 mg/kg body weight (EEN+PACs)) (n=12). Animals were anesthetized by

intraperitoneal injection of ketamine (100 mg/kg) and acepromazine (10 mg/kg). Catheters were

tunneled subcutaneously from the gastrostomy site over the back and exited mid tail. Mice were partially immobilized by tail fixation to protect the catheter during infusion. This technique does not induce significant physical or biochemical stress as was previously shown [28].

Catherized mice were connected to infusion pumps and allowed recovery for 48 hours while receiving 4 mL/day saline (0.9%) and *ad libitum* chow (Agway Inc., Syracuse, NY) and water. Following the recovery period experimental diets were given. Chow mice continued to receive 0.9% saline at 4 mL/day as well as *ad libitum* chow and water throughout the study. The EEN solution includes 6.0% amino acids, 35.6% dextrose, electrolytes, and multivitamins, with a non-protein calorie/nitrogen ratio of 126.1 (527.0 kJ/g Nitrogen). This value meets the calculated nutrient requirements of mice weighing 25 to 30 g [29]. EEN and EEN + PAC fed mice received solution at 4 mL/day (day 1), 7 mL/day (day 2) and 10 mL/day (days 3-5) as well as *ad libitum* water throughout the study.

After 5 days of feeding (7 days post-catherterization), mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and acepromazine (10 mg/kg), and exsanguinated via left axillary artery transection. The small intestine was removed and the lumen rinsed with 20 mL Hanks Balanced Saline Solution (HBSS, Bio Whittaker, Walkersville, MD). The luminal rinse was centrifuged at 2,000 x g for 10 min and supernatant aliquots were frozen at -80°C for sIgA analysis. Tissue samples were taken by removing a 3 cm segment of ileum excluding PPs. PP lymphocytes were assessed by counting on a hemocytometer. Samples were frozen in liquid N_2 and stored at -80°C until processing or fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, and stored at 4°C for immunohistochemistry.

Peyer's Patch Lymphocytes

The Peyer's patch (PP) from the entire length of the SI were removed into 1.5 mL tubes of CMF-HBSS. PP were strained through 100-μm mesh with a total volume of 15 mL CMF-HBSS. The effluent was collected and spun at 1700 rpm at 5°C for 10 min. The supernatant was removed and the pellet resuspended in 15 mL CMF-HBSS; this step was repeated. Cells were counted on a hemocytometer with trypan blue.

Tissue Cytokine Quantitative Analysis

The flash-frozen small intestine segment from each animal was homogenized in RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1% protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO). The homogenate was kept on ice for 30 min prior to centrifugation at 16,000 x g for 10 min at 4ºC. The supernatant was then stored at −20ºC until analysis. Prior to storage, the protein concentration of the supernatant was determined by the Bradford method using BSA as a standard.

Concentration of IL-4 was determined in the supernatant using solid phase sandwich ELISA kits (BD Biosciences, San Diego, CA), according to manufacturer's instructions. The absorbance at 450 nm was determined using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The IL-4 concentrations in the samples were determined by using a 4-parameter logistic fit standard curve (SOFTmax PRO software; Molecular Devices; Sunnyvale, CA) and normalized to total tissue protein content.

JAK-STAT Profiling by the JAK-STAT Antibody Microarray

The Phospho Explorer antibody microarray (Full Moon Biosystems Inc, Sunnyvale, CA), contains 42 antibodies. Each of the antibodies has six replicates that are printed on coated glass microscope slide, along with multiple positive and negative controls. The antibody array experiment was performed according to established protocol [30]. In brief, ileum tissue lysates (n=8/group) were biotinylated with Antibody Array Assay Kit. The antibody microarray slides were first blocked in a blocking solution for 30 min at room temperature, rinsed with Milli-Q grade water for 5 min, and dried with compressed nitrogen. The slides were then incubated with the biotin-labeled cell lysates (~80 µg protein) in coupling solution at room temperature for 2 hours. The array slides were washed 5 times with 1X Wash Solution and rinsed extensively with Milli-Q grade water before detection of bound biotinylated proteins using Cy3-conjugated streptavidin. The slides were scanned on a GenePix 4000 scanner and the images were analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). The fluorescence signal of each antibody was obtained from the fluorescence intensity of this antibody spot after subtraction of the blank signal (spot in the absence of antibody), and we used the signal of the phosphorylated protein to GAPDH housekeeping protein expression.

Analysis of pIgR expression by Western Blot

Solubilized protein from small intestinal tissue homogenate was denatured at 95°C for 10 min with sodium dodecylsulfate and β-mercaptoethanol, and 20 µg of protein from each sample was separated in a denaturing 10% polyacrylamide gel by electrophoresis at 150V for 1 hour at room temperature. Proteins were transferred to a PVDF membrane, and western blot was performed as previously described [31]. Densitometric measurements of protein bands were

analyzed and quantified with the NIH Image J software. pIgR standard (Cat 2800, R&D, Minneapolis, MN) was used to compare multiple gels. The combined value of the 120 kda and 94 kda bands was determined for the quantitation of the pIgR protein expression in sample.

Analysis of IgA by Western Blot

Luminal wash IgA was measured by western blot since we observed the addition of PACs to control animal luminal wash samples rapidly decreases sensitivity and total signal measured by IgA ELISA (unpublished observation), likely through the complexation between PACs with proteins [32]. 4 µL of luminal fluid was denatured at 95°C for 10 min with sodium dodecylsulfate and β-mercaptoethanol. Proteins were separated in a denaturing 10% polyacrylamide gel by electrophoresis at 150 V for 1 hour at room temperature and transferred to polyvinylidene fluoride membrane using tris-glycine buffer plus 20% methanol at 80 V for 50 min at 4°C. The membrane was blocked with 5% nonfat dry milk prepared in TBS-Tween for 1 hour at room temperature with constant agitation. Membranes were incubated with goat anti-mouse IgA, α-chain specific (Sigma-Aldrich, St. Louis, MO) diluted 1:7,000 for 1 hour at room temperature with constant agitation. Then, membranes were washed and incubated with stabilized donkey anti-goat IgA-HRP conjugated secondary diluted 1:20,000 for 1 hour at room temperature. After washing, membranes were incubated with HRP substrate (Super Signal West Femto maximum sensitivity substrate; Pierce, Rockford, IL) for 5 min and bands were detected using photographic film. Densitometric measurements of immunoglobulin α-chain protein bands (~55 kDa) were analyzed and quantified with the NIH Image J software. IgA heavy chain standard (M-1421, Sigma-Aldrich) was used to normalize across multiple gels.

Statistical analysis

Experimental values were compared using analysis of variance (ANOVA) and Fisher protected least significance difference (PLSD) corrected for multiple comparisons, with $\alpha = 0.05$ considered significant (Statview 5.0.1, SAS, Cary, NC). Numerical results are presented as mean $\pm$ standard deviation of the mean.

RESULTS

PAC characterization by HPLC and MALDI-TOF MS

The cranberry presscake PAC eluted as two unresolved peaks that had absorbance at 280 nm and minor absorbance at 520 nm due to the presence of covalently linked anthocyanin-proanthocyanidin pigments. No peaks were observed with an absorbance max typical of the other classes of cranberry polyphenolic compounds (anthocyanins, hydroxycinnamic acids, and flavonols). The poorly resolved chromatogram at 280 nm is due to structural heterogeneity of cranberry presscake PAC [27].

Reflectron mode MALDI-TOF MS showed masses that correspond to PAC with at least 1A-type interflavan bond in trimers to undecamers. MALDI-TOF MS linear mode spectra had m/z peaks that correspond to cranberry presscake PAC with a range of 3 to 23 degrees of polymerization. The spectra also contained m/z peaks that correspond to covalently linked anthocyanin-proanthocyanidin molecules, ranging from monomers to heptamers (data not shown).

Peyer's Patch Lymphocytes

Compared with Chow ($4.533 \times 10^6 \pm 1.226 \times 10^6$ cells), EEN significantly lowered PP lymphocytes ($2.428 \times 10^6 \pm 0.574 \times 10^6$ cells, $P < 0.0001$) [Figure 1]. Compared with EEN alone, PP lymphocytes were significantly higher in EEN+PAC ($3.957 \times 10^6 \pm 1.291 \times 10^6$ cells, $P < 0.001$). There were no significant differences between Chow and EEN+PAC ($P = 0.19$).

Ileum Tissue IL-4

Compared with Chow (6.5 ± 1.11 pg/mg protein), EEN significantly lowered ileum IL-4 (4.15 ± 1.44, $P < 0.01$) [Figure 2]. Compared with EEN alone, ileum IL-4 was significantly higher in EEN+PAC (5.8 ± 2.2, $P < 0.05$). There were no significant difference between the level of ileum IL-4 between Chow and EEN+PAC ($P = 0.42$).

Ileum Tissue Phosphorylated STAT6

Phosphorylated STAT6 (PSTAT6) was measured at two phosphorylation sites, Tyrosine 641 (Tyr641) and Threonine 645 (Thr645), and normalized to GAPDH expression. Compared with Chow (8.66 ± 1.5 PSTAT6 (Tyr641)/GAPDH), PSTAT6 at Tyr641 site was significantly reduced with EEN (6.08 ± 1.3, $P < 0.001$). The addition of PAC to EEN significantly elevated PSTAT6 at Tyr 641 (8.11 ± 0.7, $P < 0.01$). There was no difference between Chow and EEN+PAC ($P = 0.37$) [Figure 3A].

Similarly, compared with Chow (8.97 ± 1.6 PSTAT6 (Thr645)/GAPDH), PSTAT6 at Thr645 was significantly lower with EEN (6.60 ± 1.0, P < 0.01). The addition of PAC to EEN significantly elevated PSTAT6 at Thr645 (7.99 ± 0.9, P < 0.05), however, there were no significant difference between Chow and EEN+PAC (P = 0.13) [Figure 3B].

Ileum Tissue pIgR

EEN (10.23 ± 5.23) lowered tissue pIgR (relative concentration/ 20 ug protein) compared with Chow (20.71 ± 7.63, P < 0.001) [Figure 4]. PAC+EEN (16.13 ± 5.97, P < 0.03) levels of tissue pIgR were significantly higher than EEN alone. There were no significant difference between Chow and EEN+PAC (P = 0.08).

Luminal sIgA

Compared with Chow (17.62 ± 6.52), the level of luminal sIgA (relative concentration/ 4 uL luminal wash) was significantly lower following EEN (10.33 ± 4.23, P < 0.001) [Figure 5]. The addition of PAC to EEN (14.67 ± 5.86, P < 0.05) significantly elevated luminal sIgA compared with EEN alone. There was no significant difference between EEN+PAC and Chow (P = 0.15).

DISCUSSION

EEN allows alimentation to patients with contraindication to normal feeding by administering a liquid diet directly into the gastrointestinal tract [33]. EEN formulas are usually used in clinical conditions involving intestinal or pancreatic inflammation [2]. Our previous work demonstrates the administration of a glucose-amino acid infusion (EEN) administered via gastrostomy decreases several aspects of GALT function, including fewer PP and lamina propria lymphocytes; reduced tissue IL-4 and IL-10; pIgR, the sIgA mucosal transport protein; and decreased levels of luminal sIgA [4-7, 34]. Unfortunately, these changes result in increased susceptibility to infection and inflammation since sIgA is the primary protective molecule of specific (acquired) immunity that is secreted onto mucosal surfaces [34, 35]. sIgA opsinizes bacteria, preventing their attachment to the mucosa [36], and reduces virulent expression in enteric pathogens [37]. Consistent with its negative effect on luminal sIgA, EEN also increases mucosal barrier permeability and decreases microbiome diversity [10, 35]. Since EEN is the only enteral formula tolerated in certain patients, EEN supplements that improve host immune and barrier function are of particular value. In this work, we investigated the effect of PACs, isolated from cranberry press cake, upon GALT function leading to the release of sIgA in the intestinal lumen.

PACs are complex oligomeric polyphenolic compounds distributed in fruits, including grapes, cranberries, and apples, and other foods and beverages such as chocolate and wine [11, 12]. Epidemiological data suggest PACs may have beneficial health effects by preventing multiple chronic diseases [38]. However, PACs do not appear to leave the gut lumen for a variety of reasons, including non-hydrolysable bonds between flavan-3-ol monomeric units and their ability to complex both dietary and endogenous proteins. Further, PAC oligomers range in degree of polymerization from 3 to 25+ and therefore have higher molecular weight than other

common plant polyphenols. Due to these characteristics, rodent models demonstrate greater than 95% of PACs remain in the intestinal lumen during transit through the gastrointestinal tract [39, 40], and a recent human study demonstrated ingested PACs do not contribute to circulating flavanol levels [41]. These observations suggest PACs may exert beneficial health effects through their interaction at the gut mucosa.

Investigations have shown that PACs and precursors exert antioxidant and non-specific antimicrobial functions in the gut [38] and are capable of palliating chemically-induced colitis and ulceration while increasing Th2 cytokines, including IL-4, in gastrointestinal tissue [42-44]. *In vitro* studies demonstrate that intra-epithelial γδ T lymphocytes from the intestine respond to PACs with activation and cell proliferation that was dependent upon the PAC degree of polymerization [45]. The importance of molecular weight has also been demonstrated *in vitro*, with blood mononuclear cells, where higher molecular weight flavonoids induced greater cytokine production, including IL-4, than correspondingly lower molecular weight fractions [46]. These observations suggest PACs may play an influential role in context of mucosal barrier physiology and immunity, but also that the characteristics of the PACs are important when investigating their effects. Accordingly, we previously characterized the PACs used in this experiment [26], which showed the PACs ranged from 3-26 degrees of polymerization. This analysis allows for the characterization and reliable reproduction of chromatographic fractions for use in experimental treatments and future studies.

The expression of pIgR is regulated through IL-4 stimulation of the nuclear factor STAT-6, a member of the JAK/STAT signaling cascade [21, 47]. STAT-6, in part, regulates luminal sIgA through regulation of the mucosal transport protein pIgR [19]. We established the

importance of STAT-6 during parenteral nutrition with lack of enteral stimulation showing that lower IL-4 levels correlated levels of phosphorylated STAT-6, pIgR, and luminal sIgA. Administration of exogenous cytokines that stimulate STAT-6 phosphorylation during parenteral nutrition significantly increased levels of pIgR expression and luminal IgA levels, suggesting a cause and effect relationship [3]. In this work, EEN decreased intestinal tissue levels of IL-4 and phosphorylated STAT-6, correlated with decreased pIgR and luminal sIgA. The addition of PACs to EEN at physiological levels (100 mg GAE/kg body weight) resulted in increased tissue IL-4, STAT-6 phosphorylation, pIgR, and luminal sIgA, supporting our hypothesis that PACs may influence health by interacting with GALT function.

Previous work demonstrated that polyphenolic supplementation, including curcumin or a polyphenolic rich diet, increases sIgA levels when added to normal diets [48, 49]. However, this is the first to demonstrate PAC supplementation may improve luminal sIgA during EEN. PACs posed a significant challenge for accurately quantifying luminal sIgA, since PACs form complexation with endogenous and dietary proteins, including immunoglobulins, through hydrophobic and hydrogen bonding interaction [32]. During our analysis we observed that the addition of small concentrations of PACs to luminal wash fluid from control animals rapidly decreased the detectable levels of sIgA via ELISA quantification (unpublished observation). For this reason, measurement of luminal sIgA in this study was achieved by first denaturing and reducing intestinal wash fluid samples with heat, sodium dodecyl sulfate, and β-mercaptoethanol and performing western blot analysis to detect the sIgA heavy chain directly. Future work with PACs should take the complexation and masking effect into consideration when investigating intestinal sIgA.

One limitation to this study is we do not have evidence of whether PACs stimulate the GALT directly, such as through PP or intra-epithelial lymphocyte interactions at the mucosa, or if changes to the gastrointestinal luminal environment during the PAC addition to EEN are responsible for GALT stimulation. Other work in our laboratory demonstrated the addition of PACs to EEN maintains microbiome diversity in the gut lumen compared to the reduced diversity that both we and others have observed in EEN alone [35]. Future work aims to determine the effect of PACs upon leukocytes from GALT compartments, including the PP, intra-epithelial space, and lamina propria to assess the potential role of these cells.

In summary, this work supports the hypothesis that decreased enteral stimulation, such as EEN or parenteral feeding, suppresses GALT function – including total PP and lamina propria lymphocytes numbers, Th2 cytokine levels, and the mucosal sIgA transport protein, pIgR - that leads to reduced luminal sIgA levels. Consistent with the hypothesis of the current study, that PACs may provide immunoprotective effects through interactions with the GALT and intestinal mucosa, the supplementation of physiological doses of PACs to EEN elevated GALT function and luminal sIgA compared to EEN feeding alone. This study suggests moderate levels of PACs may be beneficial when added to enteral diets by promotion of adaptive immune function.

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Figure 1

Peyer's Patch Lymphocytes
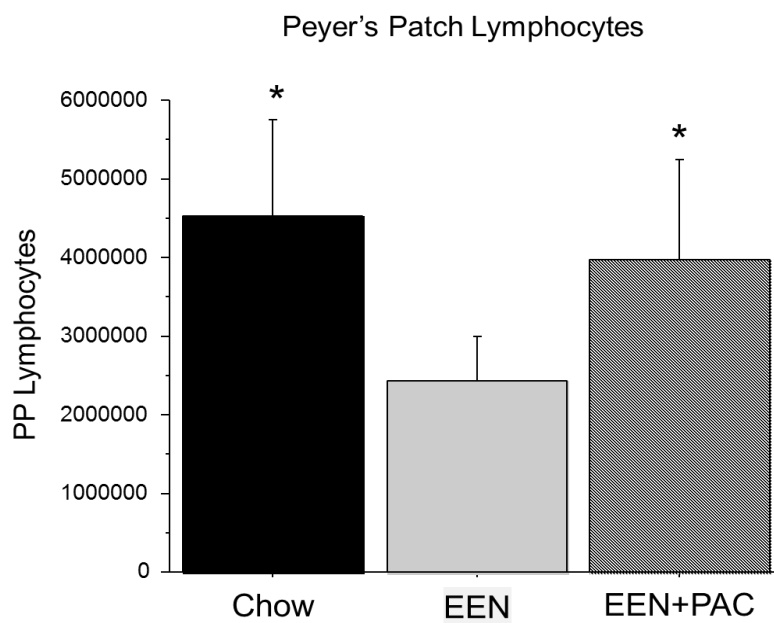
The total number of Peyer's Patch lymphocytes in Chow, EEN, and EEN+PAC fed mice. * P < 0.001 vs EEN.

Figure 2
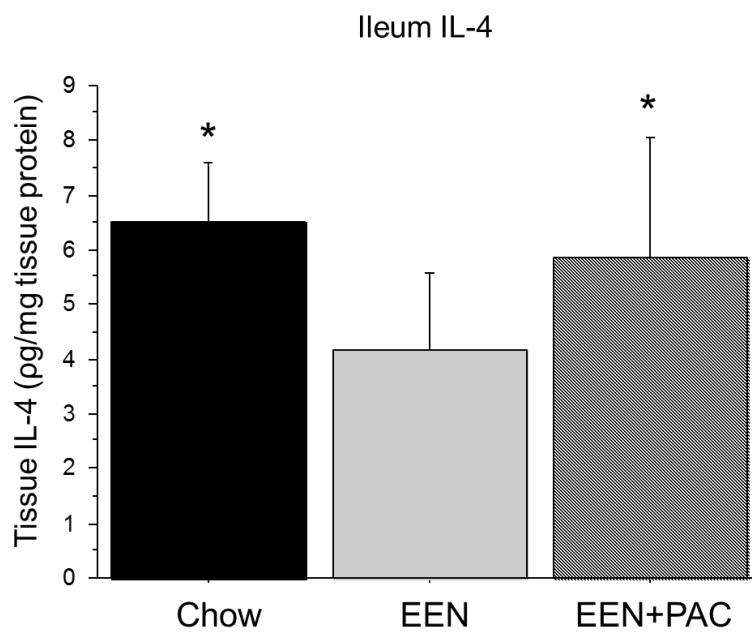
Ileum tissue IL-4 levels in Chow, EEN, and EEN+PAC fed mice. * P < 0.05 vs EEN.

Figure 3
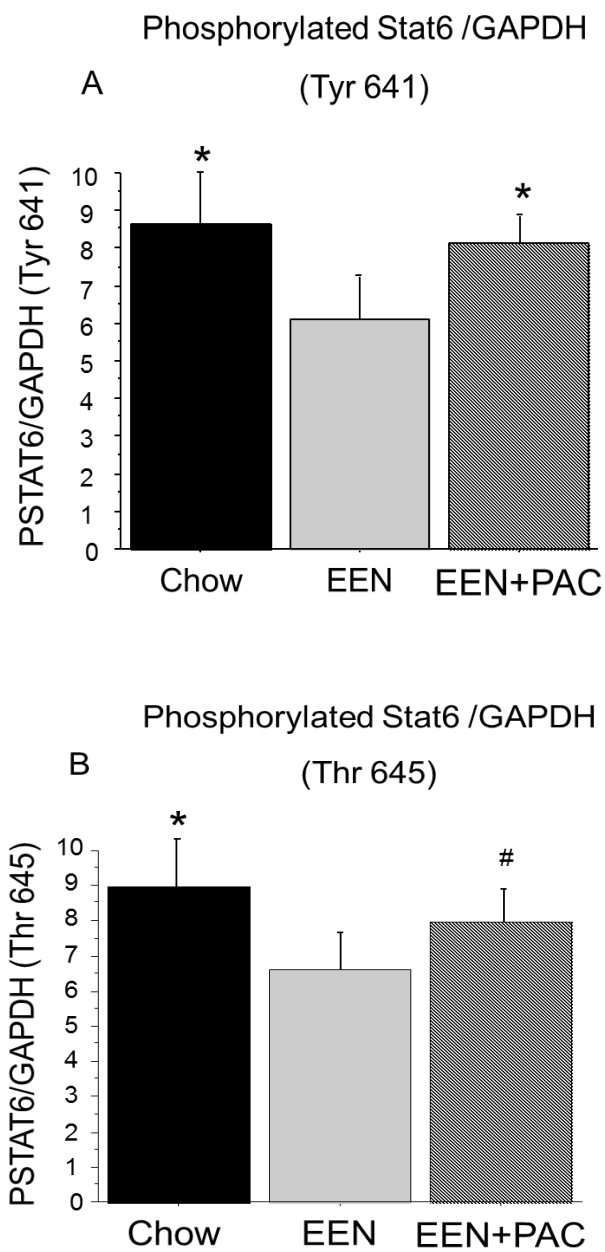
(A) Phosphorylated STAT6 (Tyr 641) ileum tissue levels in Chow, EEN, and EEN+PAC fed mice. (B) Phosphorylated STAT6 (Tyr 645) ileum tissue levels in Chow, EEN, and EEN+PAC fed mice. * P < 0.01 vs EEN. # P < 0.05 vs EEN.

Figure 4
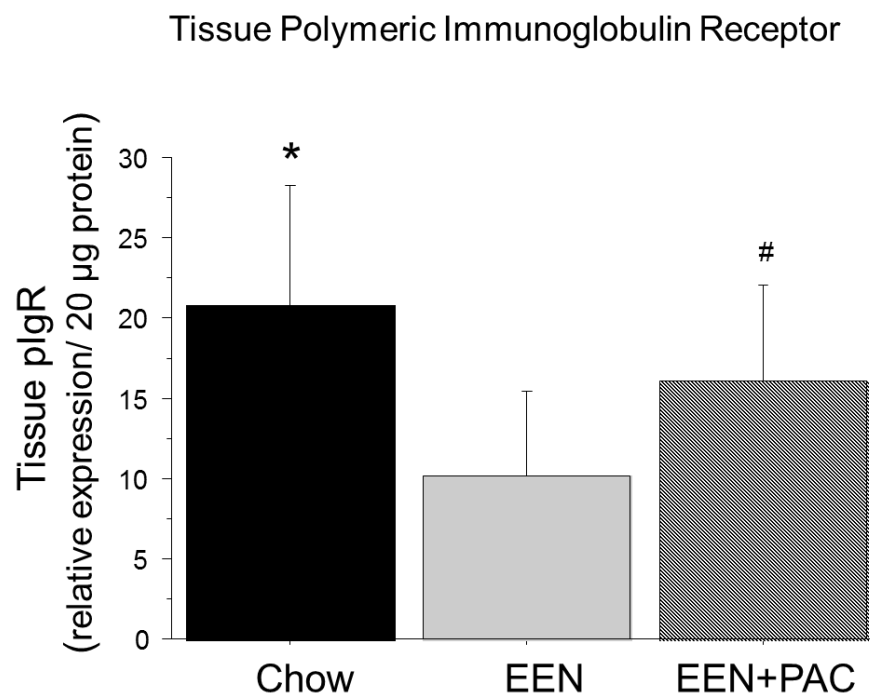
Ileum tissue levels of polymeric immunoglobulin receptor (pIgR) in Chow, EEN, and EEN+PAC fed mice. * P < 0.05 vs EEN. # P < 0.05 vs EEN.

Figure 5
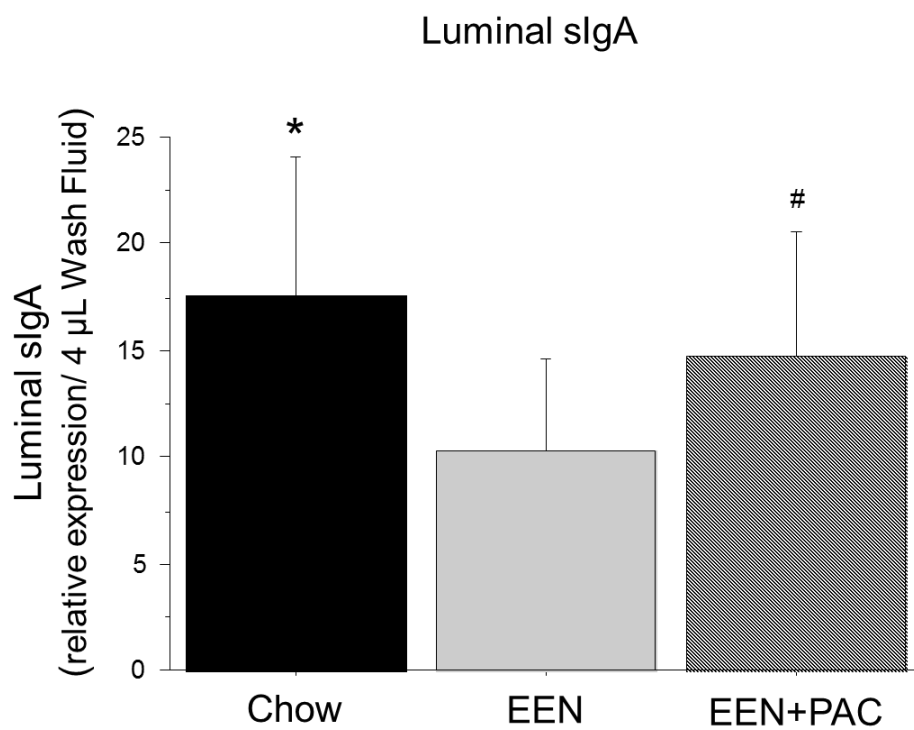
Concentration of secretory IgA in small intestine luminal wash samples in Chow, EEN, and EEN+PAC fed mice. * P < 0.01 vs EEN. # P < 0.05 vs EEN.

Chapter 6

Cranberry Proanthocyanidins Preserves Microbiome Diversity during Elemental Enteral Nutrition

Unpublished Data

INTRODUCTION

The human gastrointestinal tract is colonized by over 1000 bacterial species present in enormous quantities that are collectively termed the microbiome [1]. The average microbiome contains 10 times the number of individual cells with over 100 times the genetic diversity than its human host, highlighting the generally underappreciated role of microbes in human homeostasis and health. The microbiome plays fundamental roles in immune stimulation and maintenance, digestion, and synthesis of vitamins and short chain fatty acids that are proposed to benefit the host and is therefore implicated in influencing health[1]. Altered microbiome structures have been identified in states of metabolic dysfunction, such as obesity, diabetes, and inflammatory bowel diseases [2, 3]. One key modulatory force over microbiome structure is dietary intake, such as the ratio of carbohydrates to protein intake, dietary fiber, and polyphenolics. Another primary modulator of the microbiome is the release of antimicrobial products from Paneth cells. Under parenteral or enteral feeding where enteral bulk and complexity is decreased, especially in the proximal bowel, reduced microbiome diversity is observed [4]. The loss of microbiome diversity is associated with reduced immune parameters in the gastrointestinal tract, including decreased barrier function [5].

In investigating the role of dietary intake upon microbiome structure, recent attention has been drawn to the role of plant products, including fiber and polyphenolics [6]. Certain fermentable fibers, such as inulin and fructooligosacharides, are known prebiotics that are proposed to modulate the intestinal microbiome composition in a way that is beneficial or protective to the host [6]. With the use of metabolomic analysis, microbiome metabolism of polyphenolic compounds is also beginning to be elucidated [7]. In contrast to some polyphenolics that are

largely metabolized before exiting the colon, proanthocyanidins (PACs) remain intact during transit through the small bowel and are metabolized to lesser degrees in the large intestine [8].

Recent animal studies in our laboratory, reported in this dissertation, have investigated the effect of supplementing elemental enteral nutrition (EEN) formulations with PACs to determine their effects upon gut health, including alterations to the epithelial barrier and GALT. In those studies, we collected samples of intestinal content from the ileal-cecal junction for crude analysis of the microbiome community via automated ribosomal intergenic spacer analysis (ARISA) to determine the effect of EEN and EEN supplemented with PACs upon bacterial diversity. ARISA is useful since the spacer between 16S and 23S rRNA genes of microorganisms are commonly varied in length and sequence but well conserved between species [9]. ARISA has been shown to highly correlate with 16S sequencing as a tool for bacterial richness estimation [9]. The following is a summary of those findings along with a report of the level of sPLA$_2$ observed in the ileum tissue and lumen following Chow, EEN, or EEN+PAC (100 mg/kg GAE PACs) feeding for 5 days, beginning 2 days after intragastric feeding.

MATERIALS AND METHODS

The '*PAC Preparation and Characterization*', '*Animals*', and '*Experimental Design*' used were the same as Chapter 4 (pages 89-92). Methods for '*sPLA2 western blot*' and '*Immunohistochemistry of tissue sPLA2*' were the same as Chapter 3 (pages 62-64). In addition, during tissue harvest luminal contents were collected with 1 mL of HBSS from the last 1 cm of small intestine and stored at -80 $^\circ$C until analysis.

ARISA

Total genomic DNA was isolated from intestinal contents using DNeasy blood and tissue

Kit (Qiagen, Hilden, Germany) and the 16S-23S rRNA spacer was amplified as previously

decribed by Fisher and Triplett [10]. Briefly, PCR reations were performed in duplicate in 20 µl

reactions with Taq DNA polymerase (Invitrogen, Carlsbad, CA), 3uM of $MgCl_2$, 2 µl 10X PCR

buffer, 0.1mM dNTP mix, nuclease-free water, and 1pmol of primers. The reaction sequence was

as follows: 3 min at 94 $°C$; 30 cycles of 1 min denaturation at 94 $°C$, 1 min annealing at 53 $°C$,

1.25 min elongation at 72 $°C$; and 10 min final elongation at 72 $°C$.

Fragment Analysis and Diversity Estimation

A peak scanner (Applied Biosystems, Foster City, CA) with capillary electrophoresis

was used to analyze total product peaks and data were represented as a fragment from at least

one bacterial phylotype. Peaks observed between 300 and 1000 base pairs with a threshold of at

least 100 fluorescent units were selected to make ARISA profiles. Data were exported to

Microsoft Excel for further analysis. Since the sum of the peaks is proportional to the total DNA

concentration, peaks at each fragment size were calculated as a relative amount of total DNA.

Peaks that did not reach 0.5% of total DNA were removed from analysis in order to minimize

false peaks that might result in overestimation of species richness. The remaining peaks were

analyzed to estimate species diversity using the Shannon's diversity index and Simpsons index

of diversity (1-D) in PAST (http://folk.uio.no/ohammer/past/), which both measure the richness

and evenness of species present in samples. Significant differences were accepted at $P < 0.05$.

Next, treatment groups were compared with the Jaccard similarity coefficient, used to

statistically compared similarity among groups. Finally, the peaks were analyzed using principal

coordinate analysis (PCoA) (PAST), which aids to visualize changes in community structure across a primary and secondary axis and 95% confidence ellipses were determined.

RESULTS

ARISA

There were 53 total peaks (detected phylotypes) observed in Chow, while only 22 peaks were observed in EEN [Table 1]. There were 40 total peaks observed in EEN+PAC. After removing peaks that did not reach greater than 0.5% of total signal, there were 32 peaks in Chow, 15 in EEN, and 25 in EEN+PAC [Figure 1]. Overall, the peaks appeared more similar between Chow and EEN+PAC and either group compared to EEN alone. This was tested across the entire data set statically using peaks greater than 0.5 % of total signal and calculating Jaccard's similarity coefficient. This similarity analysis revealed that the peak profiles of Chow and EEN+PAC are 65% similar to one another, while both dietary groups are only about 50% similar to EEN alone [Figure 2]. To compare diversity, we calculated the Shannon's diversity index (SH) and Simpsons index of diversity (SI) between each group. Compared with Chow (SH 3.8; SI 0.891), EEN significantly reduced diversity (SH 2.56, $P < 0.01$; SI 0.85, $P < 0.05$). Compared with EEN, the addition of PACs to EEN significantly increased diversity (SH 3.46, $P < 0.01$; SI 0.91, $P < 0.05$). When comparing Chow to EEN+PAC, diversity differences were detected with SH ($P < 0.05$) but not SI ($P = 0.19$). Finally, PCoA revealed slight shifts in composition using Unifrac distance metrics, but striking differences were observed since 95% confidence intervals overlapped.

Ileum Tissue and luminal sPLA$_2$ levels

Immunohistochemistry visually demonstrated reduced levels of sPLA$_2$ in EEN compared with Chow, effects that were prevented with the addition of PACs to EEN [Figure 4].Compared with Chow (668 ± 262 sPLA$_2$ relative density), the relative density of intestinal rinse luminal wash samples from EEN (381 ± 87, P < 0.02) was significantly decreased [Figure 5]. Comapred with EEN alone, the addition of PACs to EEN (696 ± 347, P < 0.02) significantly increased the relative density of sPLA$_2$ in the lumen.

DISCUSSION

The relationship between diet, microbiome composition, and health is receiving more attention as a testable hypothesis following the development of methods able to investigate the complex composition of the intestinal microbiome and high-throughput expression of host genes [7, 11]. Polyphenolics are a prime target for these investigations since epidemiologic studies have repeatedly identified greater polyphenolic intake with lower incidence of chronic diseases [12]. In this study, we employed a crude method of microbial analysis, ARISA, to determine the effects of EEN or EEN supplemented with PACs upon microbiome diversity. We used ARISA since this method is cost effective and correlates well with more detailed sequencing methods to determine species diversity across samples [9]. Consistent with previous work using EEN [4], we observed a decrease in microbiome diversity compared with Chow feeding. Interestingly, the addition of PACs to EEN resulted in a partial maintenance of diversity compared with EEN alone. Since regulation of the microbiome is influenced by the release of antimicrobial compounds from Paneth cells, including sPLA$_2$, Lysozyme P, RegIIIγ, and cryptidins, we utilized western blot and immunohistochemistry methods to detect sPLA$_2$ in the luminal and tissue comparements with the experimental diets. In both the lumen and tissue we observed EEN

results in decreased levels of sPLA$_2$, consistent with Chapter 2 of this dissertation, and the

addition of PACs to EEN prevented these alterations. These findings suggest that PACs may

support microbiome structure as dietary compounds. It remains unclear if the effect upon

microbiome diversity was dependent upon PACs interaction with the bacteria, epithelium, or

both. These data build upon Chapters 4 and 5 of this dissertation that demonstrate beneficial

effects of PAC supplementation to EEN upon the intestinal mucosal barrier and GALT function.

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Table 1.

Group	Numbers of phylotypes present	Numbers of phylotypes present greater than 1%	Numbers of phylotypes present greater than 0.5 %
Chow	53	33	32
EEN	22	16	15
EEN+PAC	40	30	25

Comparison of observed peaks (estimated phylotypes) from, greater than 1%, and greater than 0.5% in ileum content samples measured via ARISA.

Figure 1.
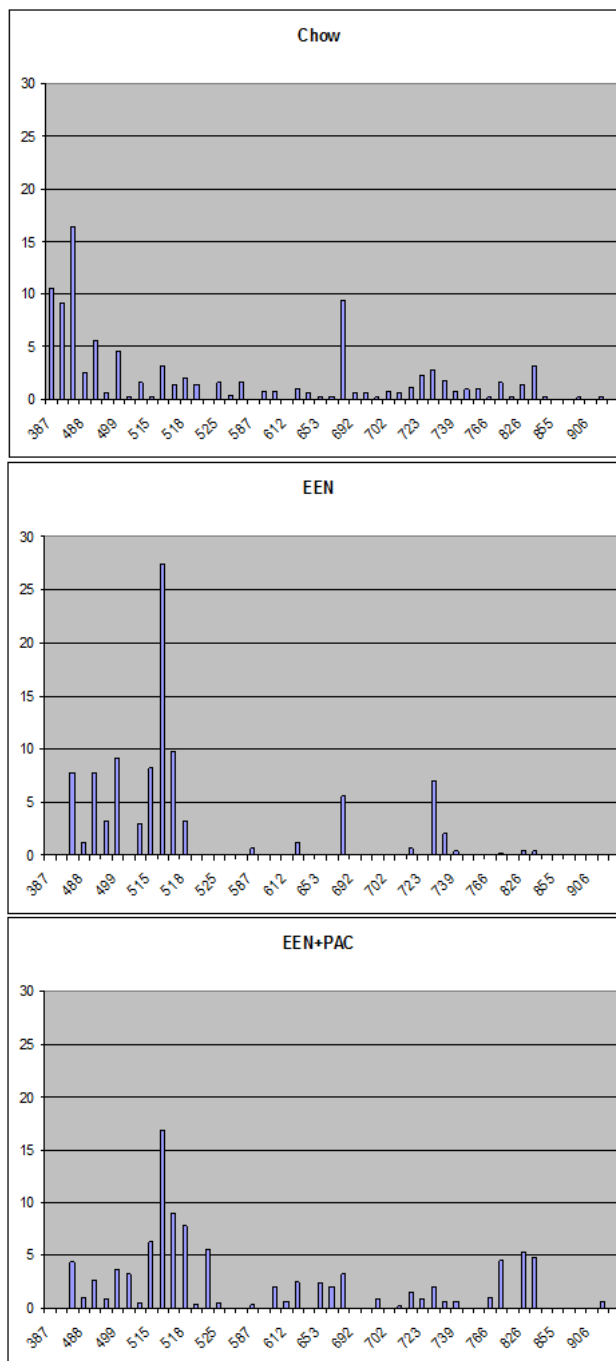
Peaks observed greater than 0.5% of total signal from ARISA.

Figure 2.
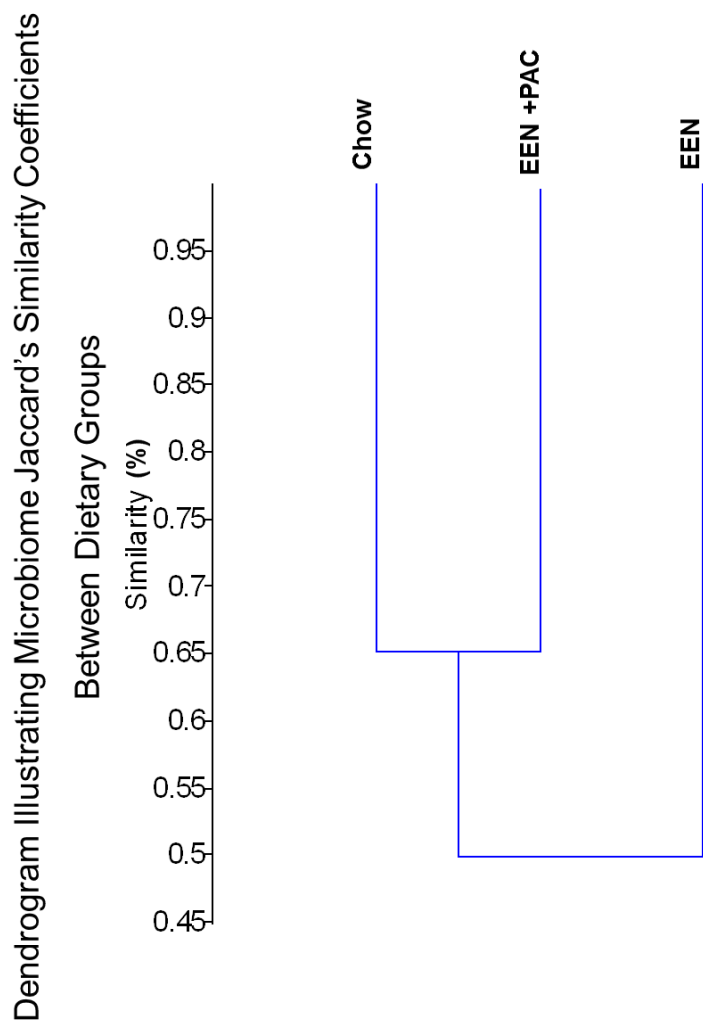
Jaccard's similarity coefficients between Chow, EEN, and EEN+PAC. The results illustrate the greatest similarity between Chow and EEN+PAC.

Figure 3.
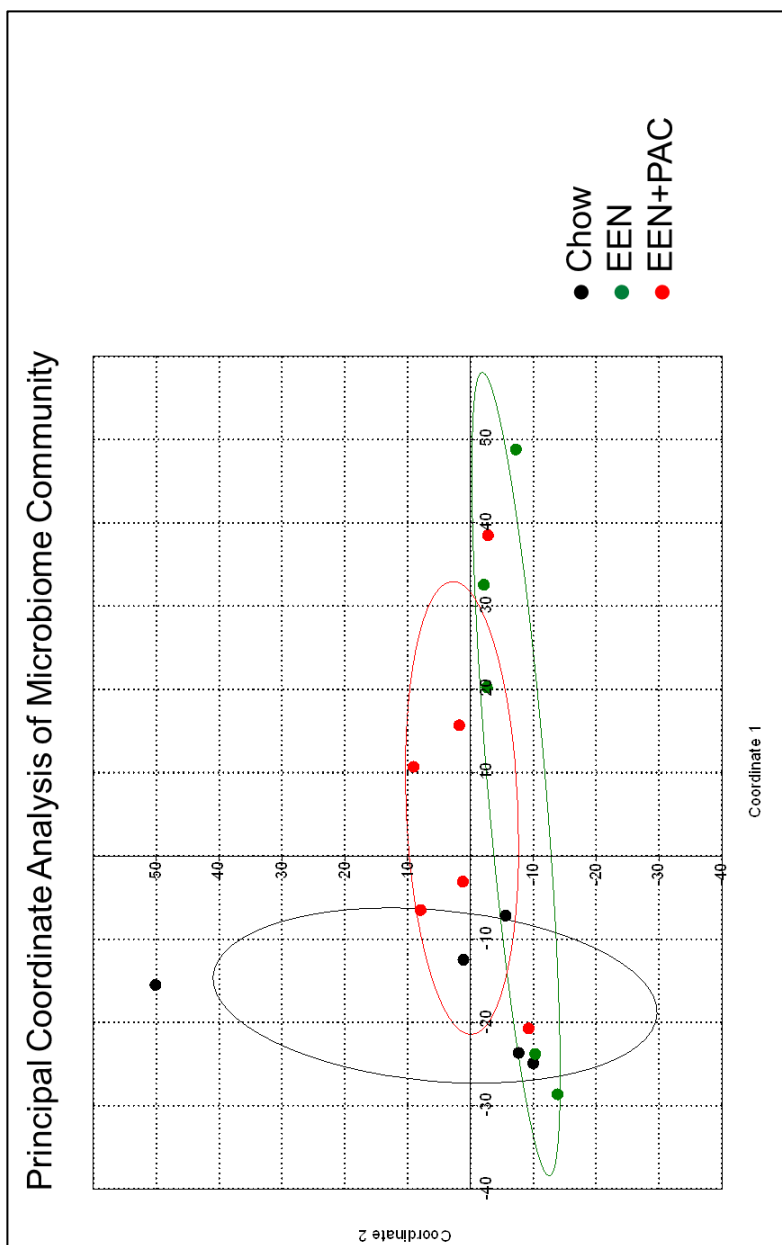
Principal coordinate analysis of Chow, EEN, and EEN+PAC dietary groups. Ellipses are 95% confidence.

Figure 4.
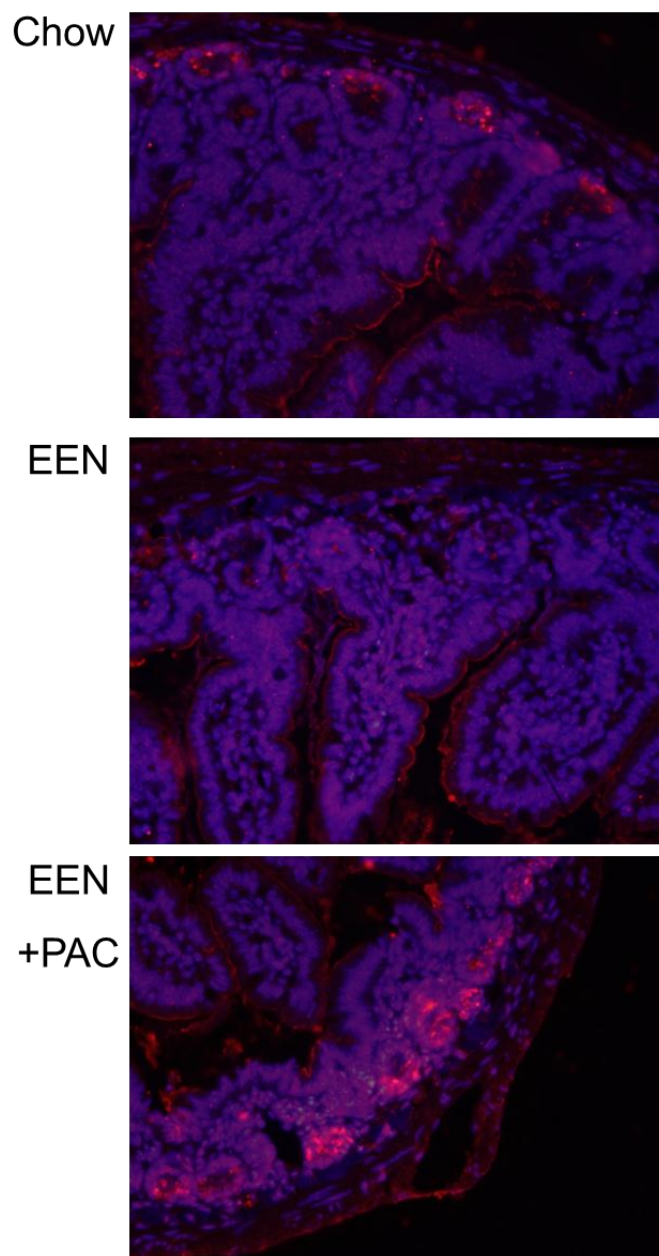
Immunohistochemistry of sPLA2 in ileum tissue from Chow, EEN, or EEN+PAC fed mice.

Figure 5.
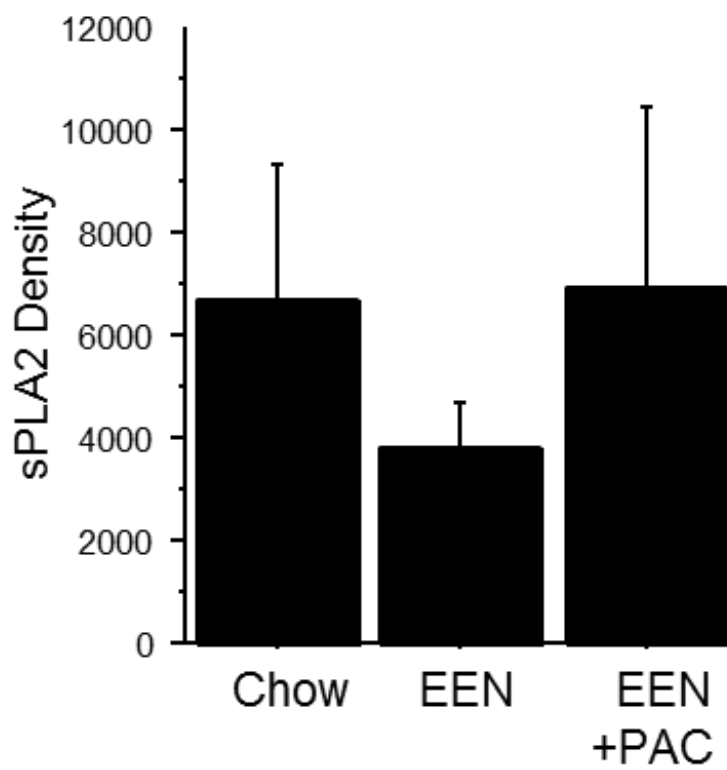
Relative density of sPLA2 in small intestinal luminal wash fluid from Chow, EEN, or EEN+PAC fed mice.

Chapter 7

IL-25 Improves IgA Levels During Parenteral Nutrition Through the JAK-STAT Pathway

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ABSTRACT

Introduction: Parenteral nutrition (PN) impairs mucosal immunity and increases infection in part via lower IgA levels at mucosal surfaces. Transport of IgA across the mucosa to the gut lumen depends on the epithelial transport protein, polymeric immunoglobulin receptor (pIgR), which is reduced during PN. *In vitro* studies demonstrate IL-4 up-regulates pIgR production via Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) signaling. Since IL-4 stimulates IgA and is reduced during PN, we hypothesized that the suppressed pIgR is a result of decreased JAK-1 and STAT-6 phosphorylation. Since IL-4 is mediated by IL-25, we also hypothesized that PN+IL-25 would restore luminal IgA by increasing phosphorylated JAK-1 and STAT-6, resulting in increased tissue pIgR and luminal IgA.

Method: Experiment 1: 2 days after IV cannulation, male ICR mice were randomized to Chow (n=11) or PN (n=9). Experiment 2: 2 days after IV cannulation, male ICR mice were randomized to Chow (n=12), PN (n=10), or PN + 0.7 μg of exogenous IL-25 (n=11) per day. After 5 days, distal ileum tissue was collected, homogenized and protein extracted for JAK-STAT expression levels using a phospho-specific antibody microarray. Tissue was homogenized to measure pIgR expression via Western blot or fixed in 4% paraformaldehyde to measure pIgR expression via immunohistochemistry. Small intestinal wash fluid was collected and IgA was quantified using ELISA.

Results: Experiment 1: PN significantly reduced phosphorylated JAK-1 and STAT-6 compared to Chow. PN also decreased the tissue levels of the Th2 cytokines, IL-4 and IL-13, as well as pIgR, and luminal IgA compared to Chow. Experiment 2: Exogenous administration of PN + IL-

25 increased the phosphorylated JAK-1 and STAT-6 compared to PN alone. IL-25 completely

restored expression of IL-13 to Chow levels. IL-4, pIgR, IgA, and phosphorylated JAK-1 were

significantly increased with IL-25 treatment compared to PN, but failed to reach levels measured

in Chow. STAT-6 was significantly increased with IL-25 treatment compared to Chow and PN.

Conclusion: PN significantly decreases the JAK-STAT pathway by reducing levels of

phosphorylated STAT-6 and JAK-1. Consistent with our previous work, sIgA, pIgR, and IL-4

decreased with PN while the addition of IL-25 to PN reversed these decreases and demonstrated

the role of the JAK-STAT pathway *in vivo* during PN.

INTRODUCTION

Parenteral nutrition (PN) is associated with an increased risk of infectious complications in the critically ill compared to enteral feeding (EN) [1-4]. Experimentally, PN alters established mucosal immune defenses by decreasing the total number of lymphocytes throughout the gut associated lymphoid tissue (GALT) including Peyer's Patches, the intraepithelial space, and the lamina propria [5]. PN also reduces levels of two IgA-stimulating Th2 cytokines including IL-4 and IL-10 in the small intestine [6], and decreases the primary adaptive immune molecule, immunoglobulin A (IgA), which is transported by epithelial cells from the lamina propria onto the mucosal surfaces [5, 7, 8]. The reduction of IgA levels following PN is, in part, through reduced production of the primary transport protein, polymeric immunoglobulin receptor (pIgR) [9, 10]. These changes in the mucosal immune system with PN are consistent with the increased risk of infection in hospitalized patients.

The Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) signaling pathway is one of the pleiotrophic cascades employed to transduce many cell signaling events and is activated by hormones, growth factors, and cytokines [11-16]. JAK/STAT provides the principle intracellular signaling mechanism required for a wide array of cytokines including IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-11, IL-13, IL-15 and IFN-γ [16-18]. Among other functions, JAK/STAT proteins influence cell development and immune stimulation [19]. Specifically, the Th2 cytokines IL-4 and IL-13 stimulate differentiation and maturation of B cells [20, 21]. Binding of IL-4 and IL-13 to an IL-4 receptor (IL-4R) initiates activation of JAK proteins through phosphorylation. Activated JAK proteins then phosphorylate a tyrosine residue on STAT-6, which otherwise remains latent in the cytoplasm. Uniquely, IL-4 and IL-13 induce tyrosine

phosphorylation and activation of STAT-6. *In vitro* work demonstrates that the activated STAT-6 forms dimmers, translocates to the nucleus where it binds specific DNA elements and activates transcription of several products, including pIgR [22-27]. Another Th2 cytokine, IL-25, provides powerful stimulatory effects to promote Th2 immunity by increasing expression of IL-4, IL-9, and IL-13 [28, 29]. Exogenous administration of IL-25 elicits a strong Th2 response *in vivo* and *in vitro*.

Since the lack of EN during PN reduces adaptive immunity by decreasing IgA, pIgR, and Th2 cytokines, we hypothesized that a lack of enteral stimulation during PN would decrease levels of phosphorylated JAK-1 (P-JAK-1) and STAT-6 (P-STAT-6) and impair adaptive immunity. We further hypothesized that administration of IL-25, a known potent stimulator of Th2 responses, during PN feeding would reverse these changes and increase levels of IgA, pIgR and other Th-2 type cytokines through increased levels of P-JAK-1 and P-STAT-6.

MATERIALS AND METHODS

Animals

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

Experimental Design

Experiment 1: PN effects on JAK-STAT signaling, Th2 cytokines, IgA, and pIgR

Male ICR mice, ages 6 to 8 weeks, were randomized to Chow (Chow, n = 9) or

parenteral nutrition (PN, n=11). Animals were anesthetized by intramuscular injection, weighed,

and underwent placement of silicon rubber catheter (0.012-inch I.D./0.025-inch O.D.; Helix

Medical, Inc., Carpinteria, CA) in the vena cava through the right external jugular vein. The

catheter was tunneled subcutaneously and existed at the midpoint of the tail. The animals were

housed individually in metabolic cages with wire floors to prevent coprophagia and bedding

ingestion and partially immobilized by tail restraint to protect the catheter during infusion. This

technique has proven to be an acceptable method of nutritional support and does not produce

physical or biochemical evidence of stress [30]. The catherized mice were connected to infusion

pumps and received saline (0.9%) at 4 mL/day and *ad libitum* chow (Agway Inc., Syracuse, NY)

and water during 48 hours of recovery. After 48 hours, Chow mice continued to receive 0.9%

saline at 4 mL/day as well as *ad libitum* chow and water. PN animals received PN solution at

rates 4 mL/day (day 1), 7 mL/day (day 2) and 10 mL/day (day 3 to 5), because a graded infusion

period was demonstrated to be necessary for the mice to adapt to the glucose and fluid loads. The

PN solution contained 6.0% amino acids, 35.6% dextrose, electrolytes, and multivitamins,

containing 1440 kcal/L and a non-protein calories/nitrogen ratio of 128:1. These values were

calculated to meet the nutrient requirements of mice weighting 25 to 30 g [31].

After 5 days of feeding (7 days post-catheterization), mice were anesthetized by

intraperitoneal injection of ketamine (100 mg/kg) and acepromazine (10 mg/kg), and

exsanguinated via left axillary artery transection. The small intestine was removed and the lumen

rinsed with 20 mL Hanks Balanced Saline Solution (HBSS, Bio Whittaker, Walkersville, MD). The luminal rinse was centrifuged at 2,000 x g for 10 minutes and supernate aliquots were frozen at -80°C, and IgA was quantified by ELISA. Tissue samples were taken by removing a 3 cm segment of ileum, excluding Peyer's patches. These samples were frozen in liquid N_2 and stored at -80°C until processing.

Experiment 2: Exogenous IL-25 and PN effects on Th2 cytokines, IgA, and pIgR

Male ICR mice, ages 6 to 8 weeks, were cannulated as in experiment 1 and randomized to Chow (Chow, n = 12), parenteral nutrition (PN, n=12), or parenteral nutrition with exogenous IL-25 (0.7 µg/mouse/day (R&D Systems) IV) for 5 days (IL-25, n=12). Tissues were harvested as previously mentioned. Ileum tissue was collected and fixed in 4% paraformaldehyde overnight at 4 C for analysis of pIgR expression using immunofluresence.

JAK-STAT Profiling by the JAK-STAT Antibody Microarray

The Phospho Explorer antibody microarray (Full Moon Biosystems, Inc. ,Sunnyvale, CA), contains 42 antibodies. Each of the antibodies has six replicates that are printed on coated glass microscope slide, along with multiple positive and negative controls. The antibody array experiment was performed according to established protocol [32]. In brief, Ileum tissue lysates were biotinylated with Antibody Array Assay Kit. The antibody microarray slides were first blocked in a blocking solution for 30 min at room temperature, rinsed with Milli-Q grade water for 3-5 min, and dried with compressed nitrogen. The slides were then incubated with the biotin-labeled cell lysates (~ 80 µg protein) in coupling solution at room temperature for 2 h. The array slides were washed 5 times with 1X Wash Solution and rinsed extensively with Milli-Q grade water before detection of bound biotinylated proteins using Cy3-conjugated streptavidin. The

slides were scanned on a GenePix 4000 scanner and the images were analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). The fluorescence signal (I) of each antibody was obtained from the fluorescence intensity of this antibody spot after subtraction of the blank signal (spot in the absence of antibody), and we used the signal of the phosphorylated protein to non-phosphorylated protein.

Analysis of Tissue IL-4 and IL-13

Small intestine segments were homogenized in RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1% protease inhibitor cocktail (P8340. Sigma-Aldrich, St. Louis, MO). The homogenates were incubated 30 minutes on ice before centrifugation at $16,000 \times g$ for 10 minutes at 4°C. All supernatants were stored at −20°C until analyzed. Protein concentrations for each homogenate were determined by Bradford dye binding method using bovine serum albumin as standard.

Concentrations of IL-4 and IL-13 were measured in the small intestinal tissue homegenate using a solid phase sandwich ELISA (BD Biosciences, San Diego, CA). Briefly, 96-well plates (Costar, 9018) were coated in 0.1 M sodium carbonate coating buffer (pH 9.5) with 100 µL per well containing antimouse IL-4 or IL-13 in a 1:250 dilution. After an overnight incubation at 4°C, the plates were washed three times with wash buffer and blocked with 200 µL of phosphate-buffered saline (PBS) containing 10% fetal bovine serum (FBS) for 1 hour at room temperature. One hundred µL of tissue homogenate or cytokine standard (BD Biosciences) were added to respective wells. After incubation for 2 hours at room temperature, plates were washed 5 times. 100 µL of a 1:250 dilution of secondary antibody was added and incubated 1 hour at room temperature. Plates were washed 5 times before streptavidin-horseradish peroxidase (SAv-

HRP) conjugate was added, and the mixture was incubated 30 min at room temperature.

Reactions were stopped by adding 50 μL of 2N H_2SO_4, and the absorbance was read at 450 nm

in a Vmax Kinetic Microplate Reader. The mass amounts were determined by plotting sample

absorbance values on a 4-parameter logistic fit standard curve, as calculated with SOFTmax

PRO software (Molecular Devices).

Analysis of pIgR expression

   Experiment 1 - Western Blot: Solubilized protein from small intestinal tissue homogenate

was denatured at 95°C for 10 minutes with sodium dodecylsulfate and β-mercaptoethanol, and

20 μg of protein was separated in a denaturing 10% polyacrylamide gel by electrophoresis at 150

V for 1 hour at room temperature. Proteins were transferred to a PVDF membrane, and western

blot was performed as previously described [9, 33, 34]. Densitometric measurements of protein bands

were analyzed and quantified with the NIH Image J software. The combined density of the ~120-

kd and ~94-kd bands were determined for the quantitation of the pIgR protein expression in each

sample.

Experiment 2 - Immunofluorescence: Since IL-25 increases intestinal smooth muscle protein, a

known side effect of this cytokine, analysis of pIgR based on protein standardization was not

appropriate. For this reason immunoflouoresence, instead of Western Blot, was used in

experiment 2 to quantify mucosal pIgR, Immunofluoresence of pIgR was performed by fixed

intestinal segments in 4% formalin overnight, processing (Tissue-Tek V.I.P), and embedded in

paraffin. Sections were cut (5 microns) and deparaffinized with heat and xylene. Samples were

rehydrated and antigen retrieval was performed with HIER (10mM Citrate buffer, pH 6.0) in a

DeCloaking Chamber for 2 minutes. Slides were rinsed and blocked with 10% BSA in PBS for 1

hour at room temperature. Sections were incubated with pIgR primary antibody (1:100, Goat anti-mouse polyclonal antibody, AF2800, R&D Systems, Minneapolis, MN) in 1% BSA with PBS for 1 hour at room temperature. Sections were rinsed and incubated with secondary antibody (1:50, Alexa Fluor 594, Donkey anti-goat, Invitrogen, Grand Island, NY) in 1% BSA with PBS for 30 minutes at room temperature. Slides were rinsed and DAPI (P36935, Invitrogen) was applied to slides to image nuclei before coverslipping. Slides were imaged on a Nikon e600 microscope using an Olympus DP70 camera. Total pIgR expression from 12-15 villi per animal was measured over total epithelial area using NIH ImageJ software.

IgA Antibody Quantitative Analysis

IgA concentration from the SI luminal fluid was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). Methods were identical to those previously published [35]. Small intestinal luminal IgA concentrations were calculated by plotting absorbance values for an IgA standard curve, which was calculated using a 4-paramater logistic fit with SOFTmax PRO software (Molecular device).

Statistical analysis

The data are expressed as means ± standard error of the mean. Statistical significance was determined using ANOVA with Fisher's protected least significant difference *post hoc test*. Differences were considered to be statistically significant at *p < 0.05*. All statistical calculations were performed with StatView (Abacus Concepts, Berkeley, CA).

RESULTS

Experiment 1: PN effects on JAK-STAT signaling

Microarray analysis of phosphorylated JAK-1 and STAT-6

Compared to Chow, PN feeding decreased the ratios of phosphorylated to non-phosphoyrlated STAT-6 (PN: 1.06 ± 0.02 vs Chow: 1.16 ± 0.02, p = 0.004) and phosphorylated to non-phosphoyrlated JAK-1 (PN: 1.23 ± 0.04 vs Chow: 1.86 ± 0.24, p = 0.04) in intestinal tissue (Figure 1).

Tissue levels of IL-4, IL-13, and pIgR

PN significantly reduced tissue levels of both IL-4 (PN: 23.4 ± 1.6 pg/mg vs Chow: 34.5 ± 2.7 pg/mg, p = 0.002), and IL-13 (pg/mg) and IL-13 (PN: 4.4 ± 0.6 pg/mg vs Chow: 8.7 ± 1.5 pg/mg, p = 0.02) (Figure 2) compared to Chow feeding. Tissue levels of pIgR (µg pIgR/40 µg tissue protein) measured by western blot were significantly depressed in PN mice compared to chow controls (PN: $0.41 \pm .08$ vs 0.75 ± 0.06, p = 0.003) (Figure 3).

Luminal levels of IgA in small intestinal wash fluid

Consistent with our previous reports, PN significantly reduced intestinal fluid IgA compared to Chow (188.1 ± 23.4 vs 387.4 ± 37.9 ng/mL , p < 0.0001) (Figure 4).

Experiment 2: IL-25 stimulation of PN

Microarray analysis of phosphorylated STAT-6 and JAK-1

Consistent with the first experiment, PN significantly decreased the ratios of phosphorylated

to non-phosphorylated STAT-6 (PN: 0.51 ± 0.02 vs Chow: 0.65 ± 0.04, p = 0.04) (Figure 5A),

and of phosphoyrlated to non-phosphoyrlated JAK-1 compared to Chow (PN: 1.1 ± 0.04 vs

Chow: 1.5 ± 0.04, p = 0.0001) (Figure 5B). PN+IL-25 significantly increased the

phosphoyrlated to non-phosphoyrlated STAT-6 ratio compared to PN alone (PN+ IL-25: $0.78 \pm$

0.07 vs. PN: 0.51 ± 0.02, p < 0.0001) and Chow (PN+ IL-25: 0.78 ± 0.07 vs Chow: 0.65 ± 0.04, p

< 0.05). PN+IL-25 significantly increased the ratio of phosphoyrlated to non-phosphoyrlated

JAK-1 compared to PN alone (PN+ IL-25: 1.2 ± 0.03 vs PN: 1.1 ± 0.04, p = 0.02) but the ratio

remained below levels observed in Chow (PN+ IL-25: 1.2 ± 0.03 vs Chow: 1.5 ± 0.04, p =

0.0004).

Tissue IL-4 and IL-13 Levels

Consistent with Experiment 1, PN significantly reduced tissue levels of IL-13 (pg/mg)

compared to chow (PN: 8.1 ± 1.0 vs Chow: 15.3 ± 1.8, p = 0.002) (Figure 6A). The addition of

IL-25 to PN significantly increased IL-13 tissue levels compared to PN alone (PN+ IL-25: $13.9 \pm$

1.6 vs PN: 8.1 ± 1.0, p = 0.005) and the IL-13 levels were simila rto Chow levels (PN+ IL-25:

15.3 ± 1.8 vs Chow: 13.9 ± 1.6, p = 0.5). Consistent with our previous studies, PN significantly

decreased the tissue levels of IL-4 compared to chow fed mice (PN: 22.1 ± 0.9 pg/mg protein vs

Chow: 34.5 ± 2.7 pg/mg protein, p = 0.0003) (Figure 6B). The addition of IL-25 to PN

significantly increased tissue IL-4 compared to PN alone (PN+ IL-25: 28.4 ± 1.8 pg/mg protein

vs PN: 22.1 ± 0.9 pg/mg protein, p = 0.03), the IL-4 levels remained lower that Chow (PN+ IL-

25: 28.4 ± 1.8 pg/mg protein vs Chow: 34.5 ± 2.7 pg/mg protein, p < 0.05).

Immunohistochemical analysis of pIgR expression in Ileum Tissue

PN significantly decreased the expression of pIgR (pIgR/total mucosa area%) as a percentage of total mucosal area in ileal tissue compared to Chow alone (PN: 0.34 ± 0.04 vs Chow: 0.61 ± 0.02, p = 0.0002). The addition of IL-25 to PN significantly increase expression of pIgR in ileal tissue compared to PN alone (PN+ IL-25: 0.51 ± 0.01 vs PN: 0.34 ± 0.04, p = 0.003), but pIgR after IL-25 treatment mice was still significantly depressed compared to chow (PN+ IL-25: 0.51 ± 0.01 vs Chow: 0.61 ± 0.02, p = 0.04) (Figure 7).

IgA concentration in Small intestinal wash fluid

As previously reported, SI luminal fluid IgA concentrations (ng/ml) significantly decreased in PN compared to Chow (PN: 163.4 ± 24.3 vs Chow: 398.1 ± 39.4, p < 0.0001). IL-25 significantly increased SI luminal fluid IgA concentrations (ng/ml) compared to PN (PN+ IL-25: 281.4 ± 29.7 vs PN: 163.4 ± 24.3, p = 0.01) but not to the levels of Chow mice (PN+ IL-25: 281.4 ± 29.7 vs Chow: 398.1 ± 39.4, p = 0.01) (Figure 8).

DISCUSSION

PN provides nutrition to patients unable to be fed enterally. Its use, however, is associated with clinical increases in infectious complications in critically ill patients. Our previous work has defined a cogent explanation for this clinical observation: an impaired adaptive mucosal immune function during PN. This impairment is multi-factorial. Compared to enteral feeding, PN decreases total GALT T & B lymphocytes [5], Th2 cytokines [6], IgA at mucosal surfaces [5, 8], and the IgA transport protein, pIgR [9, 36]. The current work expands on our previous observations by examining the JAK/STAT pathways.

The JAK/STAT pathways regulate the IgA transport protein, pIgR. STAT6 transcription stimulates production of intestinal pIgR. STAT6 transcription is generated by the Th2 cytokines, IL-4 and IL-13. These cytokines utilize a common receptor and signal solely through phosphorylation of JAK-1 and STAT-6. Since our prior works showed intestinal IL-4 drops during PN, this work examined changes in phosphorylation of tissue JAK-1 and STAT-6 proteins during PN feeding and confirmed a reduction in phosphorylation. We showed PN that was associated with reductions in phosphorylated JAK-1 and STAT6 compared with enteral feeding. Consistent with our previous work, PN resulted in lower levels of tissue IL-4, pIgR, and luminal IgA. We also observed, for the first time, that PN significantly reduced the Th2 cytokine, IL-13, in the intestinal tissue. These data supported our working hypothesis that PN diminishes established adaptive immunity by decreasing levels of phosphorylated JAK-1 and STAT-6, which are necessary mediators of pIgR transcription. However, these observations do not establish a direct causal relationship between the Th2 cytokines and pIgR mediated IgA protection.

Our second experiment confirmed a causal relationship between the JAK/STAT pathway and pIgR production by stimulating the STAT6 pathway with exogenous IL-25. IL-25 is a powerful Th2 cytokine that promotes production of IL-4 and IL-13 both *in vitro* and *in vivo* [28, 29]. We confirmed IL-25 increased tissue levels of IL-4 and IL-13 as well as expression of the phosphorylated JAK-1 and STAT-6 proteins resulting in increased tissue pIgR and luminal sIgA. Addition of IL-25 to PN increased intestinal IL-4, IL-13, phosphorylated JAK-1, phosphorylated STAT-6, pIgR, and luminal IgA compared to PN alone. These results established a direct relationship between the JAK-STAT signaling pathway with the Th2 cytokines IL-4 and IL-13 and the effector functions of adaptive immunity i.e. IgA levels in the intestinal fluid.

Interestingly, IL-25 significantly improved JAK-1, IL-4, IgA, and pIgR compared to PN alone, but not all of these parameters were restored to Chow levels. This partial restoration indicates involvement of other stimuli and signaling pathways in the production of IgA and the epithelial transport protein, pIgR. For example, our prior work showed that IL-10, another Th-2 cytokine important in IgA production, decreases with PN; there is no evidence that IL-25 stimulates release of this cytokine. IL-25 does exert its effects on other pathways separate from the Th2 cytokines. For example, exogenous administration of IL-25 causes proliferation of smooth muscle actin [37, 38], an effect demonstrated in our microarray analysis. This mandated the use of different methods for quantification of pIgR in the two experiments, as detailed in the methodology. The IL-25 stimulated smooth muscle hypertrophy elevated the house keeping proteins normally used for Western blot analysis. Therefore, to appropriately quantify pIgR in experiment 2, we used immunofluoresence imaging of the mucosa and measured pIgR expression levels directly. Thus, our partial restoration of adaptive parameters may be a consequence of the actions of IL-25 on other signaling pathways which were not measured in this work.

There are several additional limitations to this study. First, we used whole intestinal tissue for our analysis and did not examine specific cell types in our analysis. Thus, we are unable to attribute the effects to a specific cellular compartment such as lamina propria or intraepithelial cell types. Instead, our results represent changes to our measured parameters for the piece of ileum tissue as a whole. Secondly, we did not examine the IL-25 effects on other mucosal events. For instance IL-25 also stimulates the Th2 cytokine IL-5 [39, 40], a cytokine that induces eosinophilic responses. In addition, others have shown IL-25 causes increased mucus production and epithelial cell hyperplasia and hypertrophy within the lung and intestinal tissue [41]. The

additional pathways stimulated by IL-25 could contribute to the partial restoration of IgA we observed in this work. Although IL-25 contributions to other cell types and tissues were beyond the scope of the current investigation, further experiments exploring the ramifications of exogenous IL-25 administration and the effects on other pathways is currently under investigation.

In summary, this study provides a mechanism for the PN- induced reduction in the IgA transport protein, pIgR. Consistent with our hypothesis, we showed that PN decreases IL-4 and IL-13, the Th2 cytokines which normally stimulate phosphorylation levels of JAK-1 and STAT-6 and documented a reduction in the JAK-STAT pathway activation. We established a causal relationship between the IgA transport system and the JAK-STAT pathway by administering IL-25 to PN fed mice. IL-25 partially restored this transport system by increasing expression of Th2 cytokines, phosphorylated JAK-1 and phosphorylated STAT-6, IgA, and the transport protein pIgR. This work demonstrates that PN-induced vulnerabilities in adaptive mucosal immunity are at least partly driven by Th2 cytokine production and release.

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Figure 1
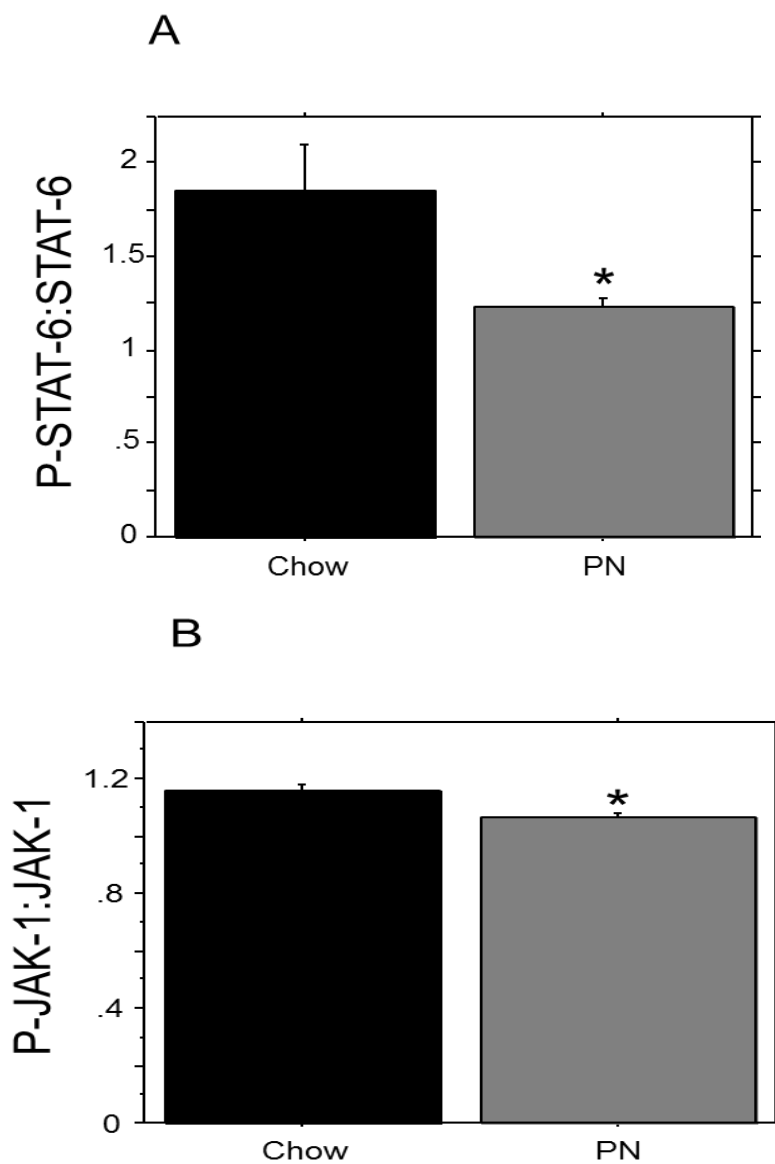
Ratio of tissue levels of phosphorylated to non-phosphorylated expression for STAT-6 and JAK-1. Parenteral nutrition (PN) significantly decreased the ratio of (A) P-STAT-6:STAT-6 and (B) P-JAK-1:JAK-1 compared to Chow. Data are represented as mean ± SEM. *$p < 0.02$ vs Chow.

Figure 2

A

B
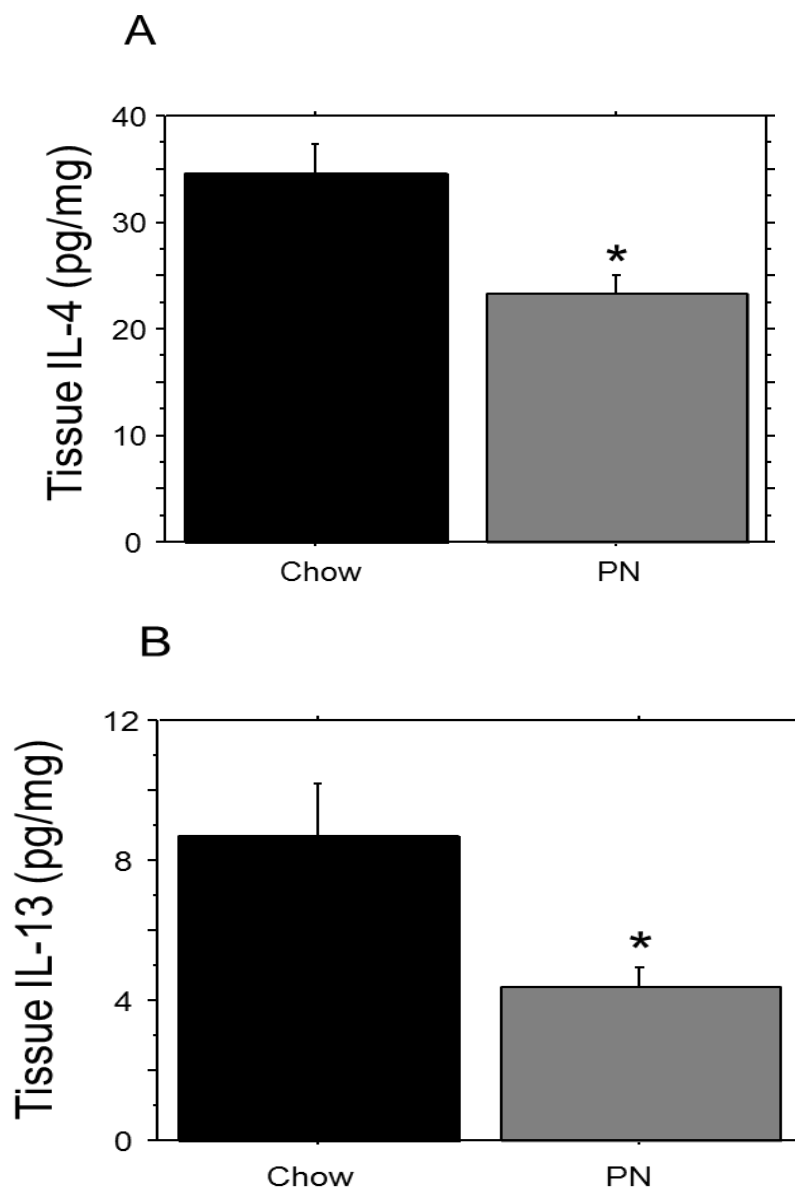
Tissue levels of IL-4 and IL-13. Intravenous parenteral nutrition (PN) significantly decreased tissue levels of (A) IL-4 and (B) IL-13 compared to Chow. Data are presented as mean ± SEM. *p < 0.04 vs Chow.

Figure 3
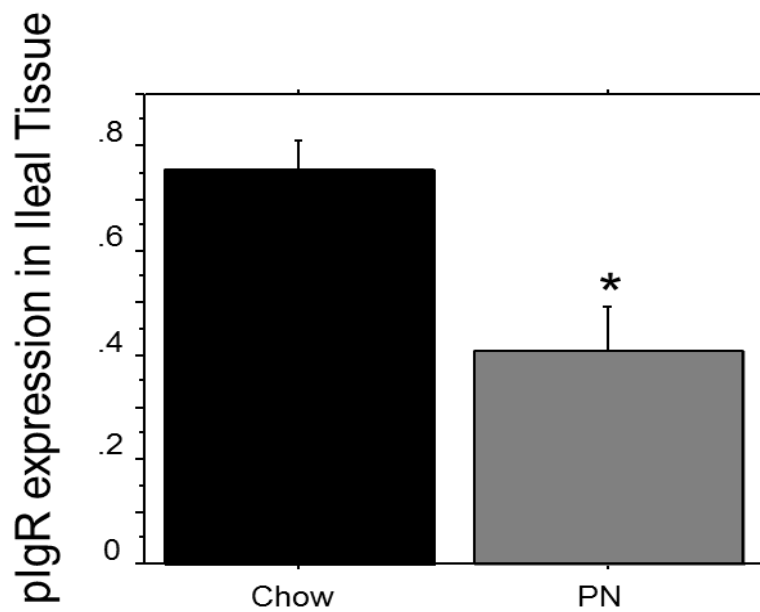
Western blot analysis of pIgR expression in Ileum tissue. Parenteral nutrition (PN) significantly decreased the expression of pIgR compared to Chow. Data are presented as mean ± SEM. *p < 0.003 vs Chow.

Figure 4
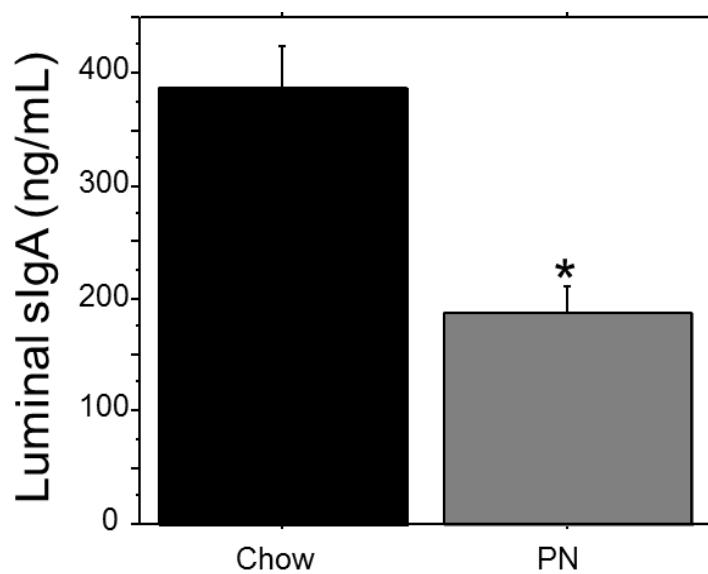
Intestinal fluid immunoglobulin A (IgA) concentration. Parenteral nutrition (PN) significantly suppressed levels of IgA compared to Chow. Data are presented as mean ± SEM.
*p < 0.0001 vs Chow.

Figure 5
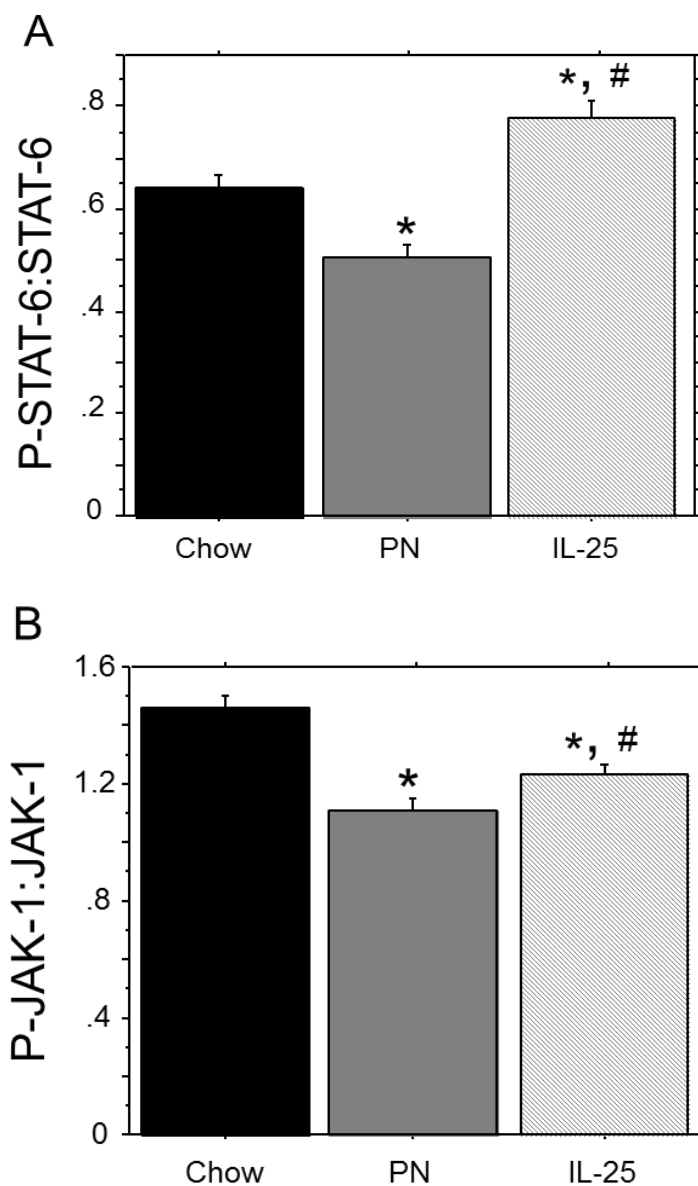
Ratio of tissue levels of phosphorylated to non-phosphorylated for STAT-6 and JAK-1 after exogenous IL-25. (A) Parenteral nutrition (PN) significantly decreased the ratio of P-STAT-6:STAT-6 compared to Chow and IL-25. The addition of IL-25 to PN significantly increased the ratio P-STAT-6:STAT-6 compared to Chow and PN. (B) PN significantly decreased the ratio of P-JAK-1:JAK-1 compared to Chow and IL-25. The addition of IL-25 to PN significantly increased the ratio of P-JAK-1:JAK-1 compared to PN, but the ratio was still significantly depressed compared to Chow. Data are represented as mean ± SEM. *p < 0.05 vs Chow. #p < 0.05 vs PN.

Figure 6
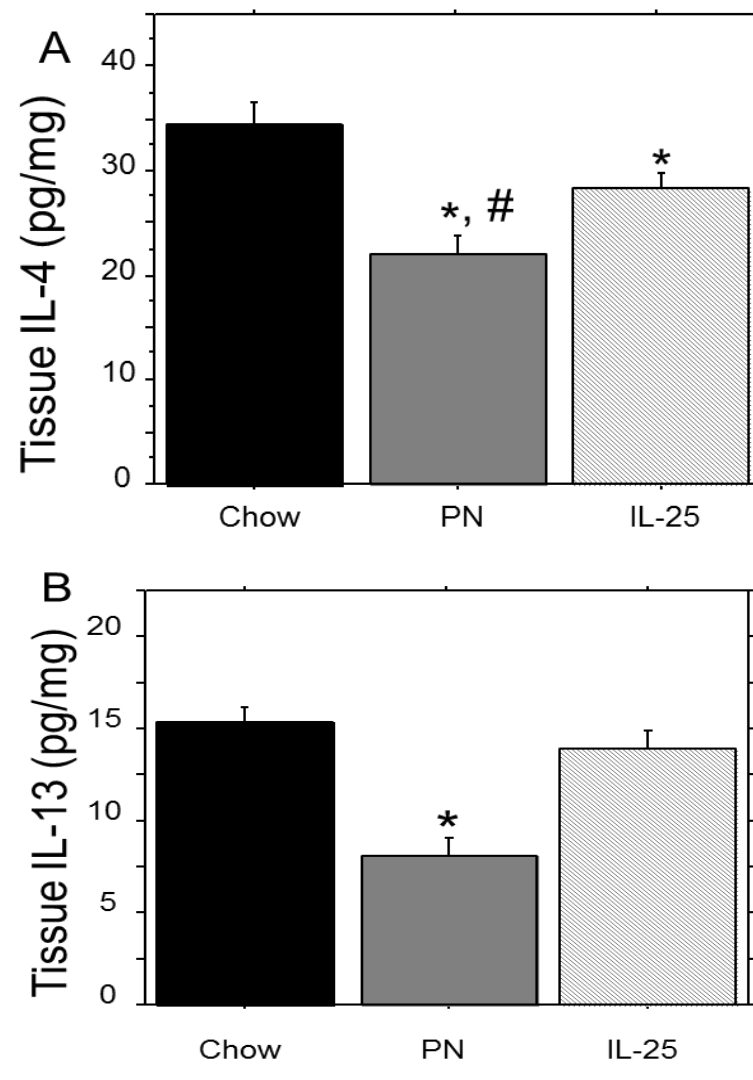
. The addition of IL-25 to PN significantly increased IL-4 compared to PN but failed to restore levels back to chow levels. (B) IL-13 was significantly decreased in PN compared to Chow and IL-25. The addition of IL-25 to PN restored IL-13 to Chow levels. . Data are presented as mean ± SEM. *p < 0.005 vs Chow.
#p < 0.03 vs IL-25.

Figure 7
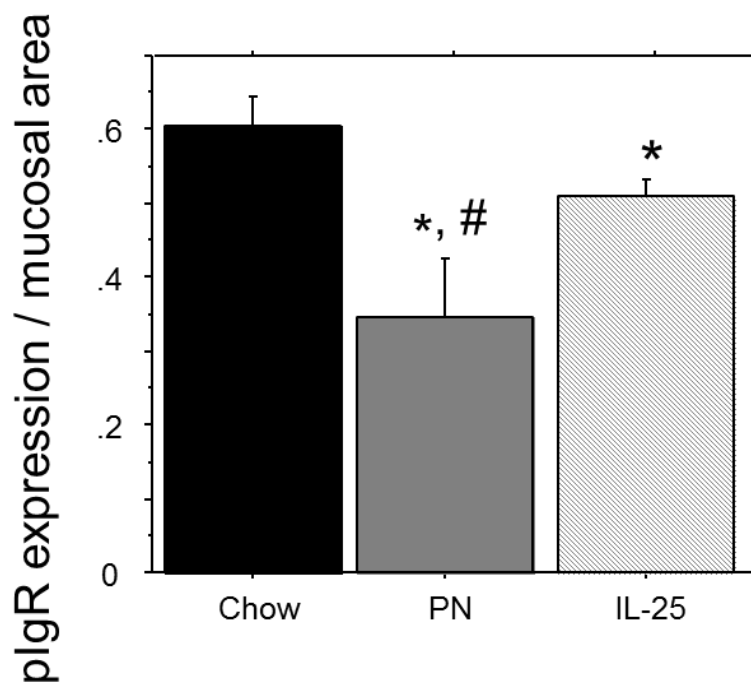
Expression of pIgR in Ileum tissue after exogenous IL-25. Parenteral nutrition (PN) reduced the expression of pIgR in ileum tissue compared to Chow and IL-25. The addition of IL-25 to PN significantly increased the expression of pIgR compared to PN, but the levels were still decreased compared to Chow. Data are presented as mean ± SEM. *p < 0.05 vs Chow. #p < 0.05 vs IL-25.

Figure 8
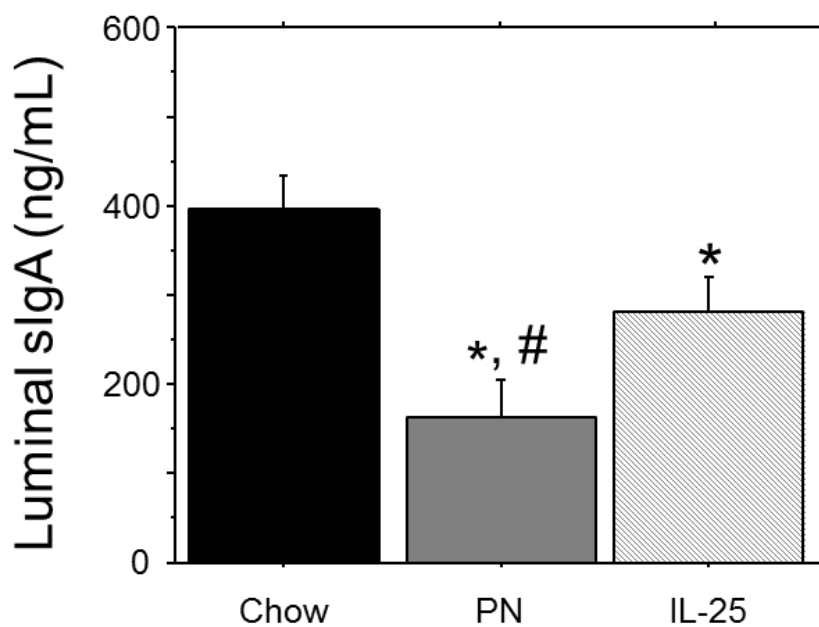
 Intestinal fluid immunoglobulin A (IgA) concentration. Parenteral nutrition (PN) significantly suppressed levels of IgA compared to chow and IL-25. The addition of IL-25 to PN significantly increased luminal levels of IgA compared to PN, but the levels are significantly decreased compared to chow alone. Data are presented as mean ± SEM. *$p < 0.05$ vs Chow. #$p < 0.05$ vs IL-25.

Chapter 8

Parenteral Nutrition Increases Susceptibility of Ileum to Invasion by *E. coli*

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ABSTRACT

Background: Parenteral nutrition (PN) compromises mucosal immune function and increases the risk of infections. We developed an *ex vivo* intestinal segment culture (EVISC) model to study the *ex vivo* effects of PN on susceptibility of the ileum to invasion by extraintestinal pathogenic *Escherichia coli* (ExPEC) and on ileal secretion of antimicrobial secretory phospholipase A_2 ($sPLA_2$) in response to the pathogen.

Materials and Methods: Study1: Using mouse (n=7) ileal tissue, we examined the effects of ileal region (proximal vs. distal) and varying ExPEC inoculum concentrations on *ex vivo* susceptibility to ExPEC invasion and $sPLA_2$ secretion. Study2: Ten mice were randomized to oral chow or intravenous PN-feeding for 5 days (n=5/group). Using the EVISC model, we compared the susceptibility of ileal tissue to invasion by ExPEC and $sPLA_2$ secretion in response to the pathogen.

Results: Study1: The proximal ileum was more susceptible to invasion (P<0.0001) and secreted lower amounts of $sPLA_2$ (P=0.0002) than the distal ileum. Study2: Ileal tissue from PN-fed animals was more susceptible (approximately 4-fold, P=0.018) to invasion than those from chow-fed animals. Ileal tissue from PN-fed animals secreted less $sPLA_2$ (P<0.02) than those from chow-fed animals.

Conclusions: The data illustrate EVISC as a reproducible model for studying host-pathogen interactions and the effects of diet on susceptibility to infections. Specifically, the findings support our hypothesis that PN decreases mucosal responsiveness to pathogen exposure and provides a plausible mechanism by which PN is associated with increased risk of infectious complication.

INTRODUCTION

The use of parenteral nutrition (PN) is associated with an increased incidence of respiratory and intra-abdominal infections in critically injured patients (1, 2). Evidence suggests that impairment of mucosal and innate immunity following PN compromises the mucosal barrier, resulting in abnormal bacterial-host interactions (3, 4). This impairment of the mucosa is multi-factorial; it includes altered tight junction proteins, reduced release of antimicrobial peptides by Paneth cells, lowered secretion of mucus by goblet cells, lower levels of luminal secretory IgA (sIgA) and increased virulence of luminal bacteria (5-8). Slowed intestinal motility (ileus) with PN also allows bacteria to migrate into the proximal intestine (9), a region that is typically sparsely colonized ($<10^4$ CFU/mL intestinal content). The treatment of these infections is often further complicated by the involvement of pathogens resistant to antibiotics (10, 11).

While clinical advances in diagnosis and therapy can identify and treat infections with certain degree of success, an understanding of host-associated vulnerabilities at the mucosal level is necessary for the development of preventive strategies. Studies utilizing cell monolayers provide mechanistic insights into how bacteria invade the tissues, but no insight into the complex *in vivo* host-pathogen interactions that occur at the mucosal interface composed of multiple cell types (12, 13). Understanding these processes in a closely modeled system would allow for insights into the susceptibility of patients on PN to infections and for the development of more effective prophylactic treatments.

More complex models such as conventional and polarized *in vitro* organ cultures (IVOC or pIVOC) (14) using human tissue from biopsies have investigated the interactions between intestinal mucosal and enteric pathogens. Specifically, pIVOC of human pediatric specimens

have been utilized to study attaching-effacing bacteria that damage mucosal surfaces. pIVOC in Ussing chambers have been use to investigate bacterial translocation across the mucosa (3, 15).

Based on the underpinnings of the pIVOC model, we developed an *ex vivo* intestinal segment culture (EVISC) model that uniquely allows high-throughput quantitative studies of bacterial invasion of the mucosal epithelium, a key step that facilitates the survival and persistence of many intestinal and extraintestinal pathogens, and their subsequent systemic translocation (16). The EVISC model also allows for investigation of innate intestinal response to bacteria in context of the secretion of antimicrobial protein such as secretory phospholipase A_2 (sPLA_2). In this work, we assessed the effect of PN on the susceptibility of murine intestinal tissues to enteroinvasion by extraintestinal pathogenic *Escherichia coli* (ExPEC) and the secretion of mucosal sPLA_2 in response to ExPEC exposure. Since PN feeding is associated with a greater risk of infection and impaired gut-derived immune responses, we hypothesized that PN feeding would result in greater enteroinvasion by ExPEC and reduced sPLA_2 secretion by the small intestinal tissue.

MATERIALS AND METHODS

Animals

All protocols were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison, and the William S. Middleton Memorial Veterans Hospital. Male Institute of Cancer Research (ICR) mice were purchased from Harlan (Indianapolis, IN) and housed 5 per microisolater-top cage in a temperature and humidity controlled environment with a 12h/12h light/dark cycle at an Association for Assessment and Accreditation of Laboratory Animal Care-

accredited conventional facility. Animals were fed a standard pelleted mouse chow (Rodent Diet 5001; LabDiet, PMI Nutrition International, St. Louis, MO) and given *ad libitum* Chow and water for 1 week prior to initiation of the study protocol.

Study 1: Effect of ileal region and ExPEC inoculum concentration on susceptibility of ileal tissue to invasion by ExPEC and sPLA$_2$ secretion by the tissue in response to pathogen exposure.

Seven male ICR mice (7–8 weeks old) were anesthetized with intraperitoneal administration of ketamine (100 mg/kg) and acepromazine (10 mg/kg), and then euthanized by exsanguinated via left axillary artery transection. The small intestine from each mouse was removed and cleaned of mesenteric fat and vascular tissue. The lumen was rinsed with 60 mL HBSS and then with 60 mL RPMI media. Ileum segments (1.5 cm) devoid of Peyer's patches were isolated, placed in RPMI solution until use in the experiment, within 30 minutes. For regionalization studies, segments from the distal ileum (adjacent to the ileal-cecal junction) and proximal ileum (15 cm proximal to "distal ileum") were exposed to varying ExPEC inoculum concentrations; enteroinvasion by ExPEC and secretion of sPLA$_2$ in response to ExPEC exposure were measured as described in detail in later sections.

Study 2: Effects of PN-feeding on susceptibility of ileal tissue to invasion by ExPEC and sPLA$_2$ secretion by the tissue in response to pathogen exposure.

Ten male ICR mice (7–8 weeks old) were anesthetized as in Study 1, weighed and catheterized by the placement of a silicon rubber catheter (0.012-inch I.D./0.025-inch O.D.; Helix Medical,

Inc., Carpinteria, CA) in the vena cava via the right external jugular vein. The catheter was tunneled subcutaneously from the neck site, over the back, finally exiting mid-tail. The mice were partially restrained by the tail for the remainder of the study to protect the catheter during infusions; this partial restraint technique does not induce significant stress in the mice (17). The mice were also housed individually in metal wire-bottomed cages to prevent coprophagia and ingestion of bedding.

The catheterized mice were connected to infusion pumps and allowed to recover for 48 h while receiving 4 mL/d of saline (0.9%) via the catheter. The mice also received *ad libitum* chow and water. This period allows for surgical recovery as previously determined by normalization of serum cytokines and food intake. Following the recovery period, the mice were randomized (n = 5 / group) to receive oral chow or intravenous PN. The chow-fed mice were given *ad libitum* chow and water, and continued to receive 0.9% saline at 4 mL/d via the intravenous catheter. The PN mice received PN solution at 4 mL/d (day 1), 7 mL/d (day 2) and 10 mL/d (days 3-5) as well as *ad libitum* water throughout the study. We previously demonstrated that a graded infusion period was necessary for the mice to adapt to the glucose and fluid loads. The PN solution includes 6.0% amino acids, 35.6% dextrose, electrolytes, and multivitamins, with a non-protein calorie to nitrogen ratio of 128:1 (1440 kcal/L). These values meet the calculated nutrient requirements of mice weighing 25 to 30 g (18). After 5 days of chow or PN feeding, the mice were anesthetized, euthanized and the intestinal segments were collected as described in Study 1. The segments of the distal ileum from chow and PN animals were exposed to an optimal ExPEC

inoculum concentration (determined in Study 1); enteroinvasion by ExPEC and secretion of

sPLA$_2$ in response to ExPEC exposure were measured as described in detail in later sections.

ExPEC Preparation

The ExPEC strain (ExPEC Strain-5011) used in our studies was isolated from a clinical fecal

sample and characterized to be genotypically representative of ExPECs. The strain expresses

both P- and type-1 fimbriae. To aid in the selection of the strain (over other strains that may be

present in the mouse tissue), the microbe was transformed to constitutively express luciferase

(Lux) using highly stable, custom low-copy plasmid, pGEN-Lux with resistance to ampicillin.

The ampicillin resistant ExPEC were cultured under static conditions in 40 mL tryptose broth

(100 µg/mL ampicillin) for 48 hours at 37ºC. An aliquot (1 mL) was taken from the surface of

the culture and used to inoculate a new culture grown under static conditions for 24 hours at

37ºC. This culturing procedure optimizes the expression of virulence factors. On the day of the

EVISC experiment, the culture was centrifuged at 1780 x g for 11 minutes to obtain a bacterial

pellet. The pellet was washed twice in 40 mL DPBS, and then re-suspended in 1 mL DPBS to

obtain the bacterial stock solution used in the experiments. The colony forming units (CFU) of

the stock solution was determined by obtaining the optical density on a spectrophotometer

(DU640B, Beckman, Brea, CA) at 450 nm wavelength and plotting it on previously established

growth curves.

Ex Vivo Intestinal Segment Culture (EVISC)

The intestinal segments were placed on a sterile surface, carefully opened apical side up, and kept moist with RPMI during all procedures. Tissue glue (Dermabond, Ethicon, Cornelia, GA) was applied lightly on one side of a polystyrene spacer (9 mm outer diameter and a 6 mm internal aperture) that was fabricated in the laboratory. The spacer was then placed onto the apical side of the intestinal segment with gentle pressure. Once the tissue glue set (approximately 10 seconds), the bonded tissue-spacer was turned over. A second spacer was lightly coated with tissue glue and placed on the serosal side of the intestinal segment. Then a light layer of tissue glue was applied to the bottom of the serosal spacer and the intestinal segment with the attached spacers was lowered into a cell culture insert (Cat 353292, 3.0 µM pore, 12-well format, BD Bioscience, NJ). Gentle pressure was applied to ensure adherence of the bottom spacer to the cell culture insert. A schematic of spacer, intestinal segment, and cell culture insert is shown in Figure 1. The entire insert containing the sandwiched tissue segment was placed into a well of a 12-well plate prefilled with 1 mL RPMI containing ampicillin (100 µg/mL).

The ExPEC inoculum were delivered in 400 µL of RPMI containing ampicillin (100 µg/mL) to obtain final concentrations of 0 (Control), 0.4, 2, 4, 20, and 40 x10^7 CFU/well in Study 1 and 4x10^7 CFU/well in Study 2. Once the inoculums were added, the plate was incubated for 1 hour at 37ºC. At the end of the incubation period, the media in each well was collected and centrifuged at 14,000 x g for 2 minutes to obtain supernatant devoid of bacteria; the supernatant was stored at -80ºC for analysis of mucosal secretions (sPLA$_2$). Then, each well was washed three times with 500 µL of DPBS. Then 700 µL of RPMI containing gentamicin (100 µg/mL) was added to each well and incubated for 1 hour at 37ºC to kill bacteria remaining in the well or adhered to the mucosal surface; at the concentration and incubation time utilized,

the gentamicin cannot enter the cells of the tissue. The RPMI + gentamicin was removed and the

tissue was washed three times with 500 µL of DPBS. The cells of the tissue segments in the

wells were lysed to release the invaded ExPEC by adding 400 µL/well of 0.1% Triton-X in

DPBS and agitating the plate on an orbital shaker (175 rpm; New Brunswick Scientific Classic

Series C1 Shaker) for 30 minutes at room temperature; at this concentration, Triton-X has no

effect on the viability of the ExPEC. Serial dilutions (10^1-10^7) of the cell lysate were made in

DPBS and plated on Luria broth (LB) agar plates containing ampicillin (100 µg/mL), and then

incubated for 18 hours at 37ºC. Enteroinvasion was assessed by enumerating CFUs of ExPEC

grown on the plates. Bacterial invasivity was calculated by dividing the total invaded CFUs by

the total inoculum CFUs.

Scanning electron microcopy

Segments of distal ileal tissue were sandwiched between spacers, attached to cell culture inserts

and placed into wells of a culture plate prefilled with 1 mL RPMI containing ampicillin (100

µg/mL) as described in previous sections. The wells were inoculated with ExPEC ($4x10^7$

CFU/well) for 1 hour at 37ºC. Then, each well was washed three times with 500 µL of DPBS.

The tissues in the wells were fixed overnight at 4°C using 2% gluteraldehyde in 0.1M phosphate

buffer. Cells were then washed twice for 10 minutes in 0.1M phosphate buffer. The tissues

sandwiched between spacers were removed from the apparatus; the spacers were left intact to

ensure that the tissues maintained their flat orientation. Tissues were incubated in step-wise

increasing concentrations of ethanol (30-100%) for specific durations of time and then placed in

the critical point dryer chamber with molecular sieve-dried ethanol. The dryer was cooled and

maintained at 10°C using CO_2. The tissues were purged of ethanol for 2 minutes and incubated with CO_2 for 10 minutes; the process was repeated twice. After another 2-minute purge, the dryer chamber was heated to 35°C, increasing the pressure on the samples to approximately 1250 psi. The pressure was slowly decreased (~100psi/min) until 0 psi was reached. The tissues were removed from the dryer and placed in a desiccator. After desiccation, the tissues specimens were platinum coated with IBS/TM200S ion-beam sputter coater (VCR Group, San Francisco, CA) to a thickness of 2.5 nm. The tissue samples were viewed and imaged on a Field-Emission Scanning Electron Microscope Hitach S-900 (Hitachi Instruments, Santa Clara, CA) at 8keV.

Determination of sPLA$_2$ activity

sPLA$_2$ activity was performed as described previously by *Tsao et al* (19), with some modification to the substrate preparation (20). This assay uses a quenched fluorophore, bis-BODIPY®-FL C$_{11}$-PC (1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-sn-glycero-3-phosphocholine (Molecular Probes, Eugene, OR), which becomes unquenched (fluoresces) when the sn-2 ester bond of is cleaved by sPLA$_2$. This method was developed as a high-throughput assay to rapidly analyze sPLA$_2$ activity. Briefly, the substrate was prepared by mixing 10 μL of bis-BODIPY-FL C$_{11}$-PC with 1 mL aliquot of phosphatidylglycerol (2 mg/mL; Sigma, St. Louis, MO) dissolved in chloroform. The choloroform was evaporated under nitrogen, and the remaining mixture of bis-BODIPY-FL C$_{11}$-PC and phosphatidylglycerol were re-dissolved in 100% ethanol, and used as substrate. The assay reaction mixture was prepared in a glass tube on ice by mixing 10 μL of substrate solution (20 μg of phospholipids) and 10 μL of sample, and then bringing up the reaction volume to 1 mL with 0.01 M Tris-HCl (pH 7.4) buffer containing 10 mM Ca^2. An aliquot (0.3 mL) of the

reaction mixture was promptly transferred in triplicate to the wells of a white polystyrene

microplate (Porvair PS White, PerkinElmer Instruments, Norwalk, CT). The microplate was

placed in a temperature-controlled (30°C) microplate reader attached to a PerkinElmer

Luminescence Spectrometer LS50B. The fluorescence intensity (FI) in each well was recorded

every 10 sec for 70 cycles at 488 nm excitation (excitation slit: 2.5 nm) and 530 nm emission

(emission slit: 5.0 nm). After the enzymatic reactions reached equilibrium temperature, the

reaction curve was fit to a second-order polynomial equation and the first-degree coefficient was

taken as the initial rate of reaction (expressed as change in FI/min/µL sample). This initial rate is

taken to be the activity of sPLA$_2$ in the sample. Blank wells containing only substrate and buffer

were used to determined non-enzymatic background activity.

Statistical analysis

A fixed effects ANOVA model was fit for each measured parameter (total invasion, invasivity

and sPLA$_2$ secretion) using the PROC MIXED function (SAS Software (Version 8), SAS

Institute Inc, Cary, NC) to test for significant effects of bacterial inoculum concentration, ileal

regionality, and/or diet. Second-order interactions between these factors were also included in

the model whenever appropriate. The correlations between observations taken between factors

were modeled using a diagonal covariance structure. For each measured parameter, the model

was fit using the untransformed data, and the residuals were evaluated to ensure that standard

ANOVA assumptions of constant variance and normality were reasonably met. Transformations

of the data were performed if required to improve adherence to these assumptions. Type III tests

were then performed to evaluate the significance of the effects of interest for each measured

parameter, and least-square means were calculated. The data are reported as least-square mean ±

standard error of mean (SEM). Statistical significance was accepted at $P < 0.05$.

RESULTS

Study 1: Effects of ileal region (proximal vs. distal ileal segments) and ExPEC inoculum

concentration on susceptibility of ileal tissue to invasion by ExPEC.

Overall Analysis: There were significant differences ($P<0.0001$) between the susceptibilities of

the two ileal regions to enteroinvasion by ExPEC (as determined by total invasion and

invasivity); The proximal ileum was more susceptible to invasion than the distal ileum (Figures

2A and 2B). The concentration of the ExPEC inoculums also had a significant effect on

enteroinvasion ($P<0.0001$ and $P=0.0001$ for total invasion and invasivity, respectively; Figures

2A and 2B). However, there were no significant interactions between the two factors, ileal

regionality and inoculum concentration.

Total invasion: The two highest concentrations of ExPEC inoculums, 20 and 40 $x10^7$ CFU/well

induced degradation of tissue and thus were excluded from further studies. In the <u>distal ileal</u>

<u>segments</u>: The total invasion produced by the ExPEC inoculum concentration of $2x10^7$ CFU/well

($7,848 \pm 46,031$ CFU of ExPEC within cells) was significantly greater than that produced by

$0.4x10^7$ CFU/well inoculum ($2,708 \pm 33,996$ CFU; $P < 0.05$; Figure 2A). The total invasion

produced by $4x10^7$ CFU/well inoculum ($61,125 \pm 33,996$ CFU within cells) was significantly

greater than those produced by either $0.4x10^7$ ($2,708 \pm 33,996$ CFU; $P < 0.0001$) or $2x10^7$ ($7,848$

$\pm 46,031$ CFU, $P < 0.01$) CFU/well inoculums. In the <u>proximal ileal segments</u>: The total invasion

produced by the ExPEC inoculum concentration of $2x10^7$ CFU/well ($50,422 \pm 46,031$ CFU of ExPEC within cells) was significantly greater than that produced by the $0.4x10^7$ CFU/well inoculum ($5,675 \pm 46,031$ CFU; $P < 0.005$). The total invasion produced by $4x10^7$ CFU/well inoculum ($381,933 \pm 46,031$ CFU) was significantly greater than those produced by either $0.4x10^7$ ($5,675 \pm 46,031$ CFU; $P < 0.0001$) or $2x10^7$ ($50,422 \pm 46,031$ CFU; $P < 0.01$) CFU/well inoculums. When comparing the two ileal regions: total invasion of the proximal region was significantly greater than that of the distal region with $2x10^7$ ($P < 0.01$) and $4x10^7$ ($P < 0.005$) CFU/well inoculums.

Invasivity: In the distal ileal segments: The invasivity of the ExPEC was significantly greater at an inoculum concentration of $4x10^7$ CFU/well (0.15 ± 0.09 %) than at $0.4x10^7$ (0.07 ± 0.09 %; $P < 0.05$) or $2x10^7$ (0.04 ± 0.12 %; $P < 0.05$; Figure 2B) CFU/well. In the proximal ileal segments: The invasivity of the ExPEC at an inoculum concentration of $4x10^7$ CFU/well (0.95 ± 0.12 %) was significantly greater than that at $0.4x10^7$ (0.14 ± 0.12 %; $P < 0.01$) or $2x10^7$ (0.25 ± 0.12 %; $P = 0.09$) CFU/well. When comparing the two ileal regions: The invasivity of the ExPEC was significantly greater in the proximal region than in the distal region with $2x10^7$ ($P < 0.01$) and $4x10^7$ ($P < 0.0005$) CFU/well inoculums.

Scanning electron microscopy: The examination of ExPEC interaction with the ileal tissue by scanning electron microscopy revealed ExPECs attaching to the apical surface of the ileal tissue via fimbriae and then initiating entry into the epithelial cells (Figure 3).

Study 1: Effects of ileal region (proximal vs. distal ileal segments) and ExPEC inoculum

concentration on sPLA$_2$ secretion by mucosal tissue in response to pathogen exposure.

Overall Analysis: There were significant differences (P=0.0002) between the secretion of sPLA$_2$

by the two ileal regions in response to ExPEC exposure; The proximal ileum produced

significantly lower sPLA$_2$ than the distal ileum in response to the pathogen (Figure 4). The

concentration of the ExPEC inoculums also had a significant effect on the secretion of sPLA$_2$

(P<0.0001). However, there were no significant interactions between the two factors, ileal

regionality and inoculum concentration.

A baseline level of sPLA$_2$ secretion by ileal tissue was detected regardless of the presence

or absence of bacteria or bacterial inoculum (Figure 4). In the <u>distal ileal segments</u>: The sPLA$_2$

secretion induced by the ExPEC inoculum concentrations of $2x10^7$ CFU/well (3019 $\pm$ 355

ΔFI/min; P<0.05) and $4x10^7$ CFU/well (2,961 $\pm$ 262 ΔFI/min; P<0.05) were significantly greater

than the baseline level (892 $\pm$ 329 ΔFI/min) secreted by the tissue. In the <u>proximal ileal segments</u>:

Only the sPLA$_2$ secretion induced by the inoculum concentrations of $4x10^7$ CFU/well (1,407 $\pm$

355 ΔFI/min; P<0.05) was significantly greater than the baseline level (689 $\pm$ 615 ΔFI/min)

secreted by the tissue. When <u>comparing the two ileal regions</u>: The baseline sPLA$_2$ secretion by

the two ileal segments did not significantly differ. However, the sPLA$_2$ secretion induced by the

distal ileal segments were significantly higher than those secreted by the proximal ileal segments

in response to 0.4, 2, and $4 x10^7$ CFU/well (P<0.0005, P=0.001, and P<0.005, respectively).

Study 2: Effects of PN-feeding on susceptibility of ileal tissue to invasion by ExPEC and sPLA$_2$ secretion by the tissue in response to pathogen exposure.

Overall Analysis: The ileal tissue from PN-fed animals was significantly more susceptible (approximately 4-fold) to enteroinvasion than those from chow-fed animals (total invasion: 84,584 ± 18,047 vs. 20,455 ± 18,784 CFU of ExPEC within cells, P=0018; invasivity: 0.21 ± 0.05 % vs. 0.05 ± 0.05 %, P=0018; Figures 5A and 5B). Ileal tissue from PN-fed animals secreted significantly lower levels of sPLA$_2$ than those from chow-fed animals (1,959 ± 552 vs. 4,161 ± 552 ΔFI/min, P < 0.02; Figure 6).

DISCUSSION

PN is a necessity for the prevention of malnutrition in patients who are unable to be fed enterally. However, its use is associated with increased risk of infection when compared to enteral feeding. PN with lack of enteral stimulation alters host immune defenses in part by changes to the gut-associated lymphoid tissue and the mucosal barrier (3, 4, 8, 21, 22). It also affects bacterial virulence within the gut lumen due to decreased nutrient availability (6, 7), decreased pH (5), and reduced bacterial diversity (23). Current evidence suggests that under such conditions, the bacterial invasion of the mucosal epithelium may to be an initial step in inducing a systemic inflammatory response that originates from the gastrointestinal system (16, 24, 25).

In patients hospitalized for 5 days or longer, the causative organisms of hospital acquired infections include facultative anaerobes, such as *Escherichia coli* (*E. coli*), *Klebsiella, Streptococcus*, and *Enterobacter* species, as well as anaerobes, such as *Bacteroides,*

Peptostreptococcus, and *Clostridium* species (26). The preservation of mucosal barrier integrity is paramount in host defense against these microbial pathogens (3), especially in the face of increasing antibiotic resistance (10).

PN not only compromises the integrity of the physical mucosal barrier, but also alters the innate molecular defense capabilities of the barrier by reducing the secretion of mucin, antimicrobial peptides and sIgA. We recently reported that PN feeding reduces levels of the Paneth cell protein, sPLA$_2$, in the small intestinal lumen (20). sPLA$_2$ plays an important role in antimicrobial defense, in addition to the wide array of other antimicrobial peptides and proteins, such as lysozymes, defensins and RegIIIγ, produced by the Paneth cells (27, 28). We and others have demonstrated the antimicrobial action of sPLA$_2$ (29-34). Due to the cationic charge on sPLA$_2$, negatively charged bacterial cell membranes attract the protein which cleaves fatty acids from the sn-2 position of phospholipid glycerol backbones on the bacterial cell wall (35, 36). This process induces membrane permeability and subsequent lysis of the microbe.

Existing cell culture models are of limited use for investigating the complex bacterial-host interactions that occur at gut mucosa, especially for elucidating the mechanisms that affect host vulnerability to infections. While these models allow the investigator to focus on specific virulent mechanisms employed by bacteria to invade the host, they exclude the complex immune responses generated by an intact host mucosal barrier in response to pathogenic bacteria (12, 13). Perhaps, more importantly, these models do not allow for the study of the effects of types and route of diet, such as PN, on mucosal immunity as it relates to susceptibility to infections.

In this work, based on the pIVOC model (14) used by *Schuller et al.*, we developed the EVISC model for studying the effects of PN on bacterial invasion of the epithelium and the

secretory response of the mucosa to pathogen exposure. Since *E. coli* are the most commonly cultured genera of bacteria in intra-abdominal abscess and since extraintestinal pathogenic *E. coli* (ExPEC), common to gastrointestinal tract, are often a significant threat of infection when displaced from the tract, we utilized a strain of ExPEC isolated from clinical fecal samples. The ExPEC strain was transformed to express luciferase and ampicillin resistance, allowing us to recover and enumerate the specific strain in our studies with intestinal segments that may contain other bacterial strains.

Our studies, in general, indicated that increasing concentrations of ExPEC inoculum, up to $4x10^7$ CFU/well, produced increasing enteroinvasion and stimulation of $sPLA_2$ secretion by the tissue. The two highest inoculum concentrations, $2x10^8$ and $4x10^8$ CFU/well, induced observable sloughing of tissue segments. The scanning electron microscopy of ExPEC interaction with the ileal tissue revealed ExPEC attaching to the apical surface of the ileal tissue via fimbriae and then initiating entry into the epithelial cells. This mechanism of invasion is commonly utilized by microbial pathogens to gain entry into non-phagocytic host cells (37, 38). The observations were also identical to what we have previously observed in our studies exploring the invasion of enterocytes (Caco-2 cells) in culture by ExPEC (data not shown). The studies of the effects of ileal regionality on bacterial invasion showed that the proximal ileum was significantly more susceptible to enteroinvasion than the distal ileum. Additionally, the proximal region secreted less $sPLA_2$ in response to pathogen exposure compared to the distal region, suggesting the differences in susceptibility of the regions to enteroinvasion may be due to a decreased immune response, such as the decreased secretion of $sPLA_2$ and possibly other antimicrobial proteins. This finding supports the view that the distal ileum is more resistant to pathogens and is consistent with the higher *in vivo* bacterial concentrations commonly reported

in the distal intestine (39). Additionally, these observations illustrate the value of the EVISC model for understanding the mechanisms for the regional differences in mucosal immunity that exist *in vivo*.

Our studies with ileal tissue from PN- and chow-fed animals showed that PN increases the susceptibility of the ileum to enteroinvasion by ExPEC; bacterial invasion of the tissue from PN-fed animals was approximately four times higher than in that from chow-fed animals. The increased susceptibility may, in part, be due to the decreased secretion of $sPLA_2$ in response to the pathogen that was observed in tissue from PN-fed animals. These observations not only suggest why PN is associated with increased risk of infection in patients, but also illustrate the value of the EVISC model for understanding the impact of diet on susceptibility to infections and for elucidating the mechanisms by which diets alter mucosal immunity in this context.

In summary, our studies illustrate the usefulness of EVISC as a reproducible model of the heterogeneity of mucosal tissue and some of the complexity of host-pathogen interactions for studying the effect of type and route of diet in context of infections. More specifically, the data supports our hypothesis that reduced enteral stimulation decreases the effective responsiveness of mucosal immunity to a pathogen exposure, and provide a potential mechanism by which PN is associated with an increased risk of infectious complications in patients. We have further studies in this model underway to examine how stimulation of individual components of the host defense, such as Paneth cell production of $sPLA_2$, lysozymes, and defensins, lamina propria production of IgA, and goblet cell production and release of mucins, influence response to a pathogen.

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Figure 1
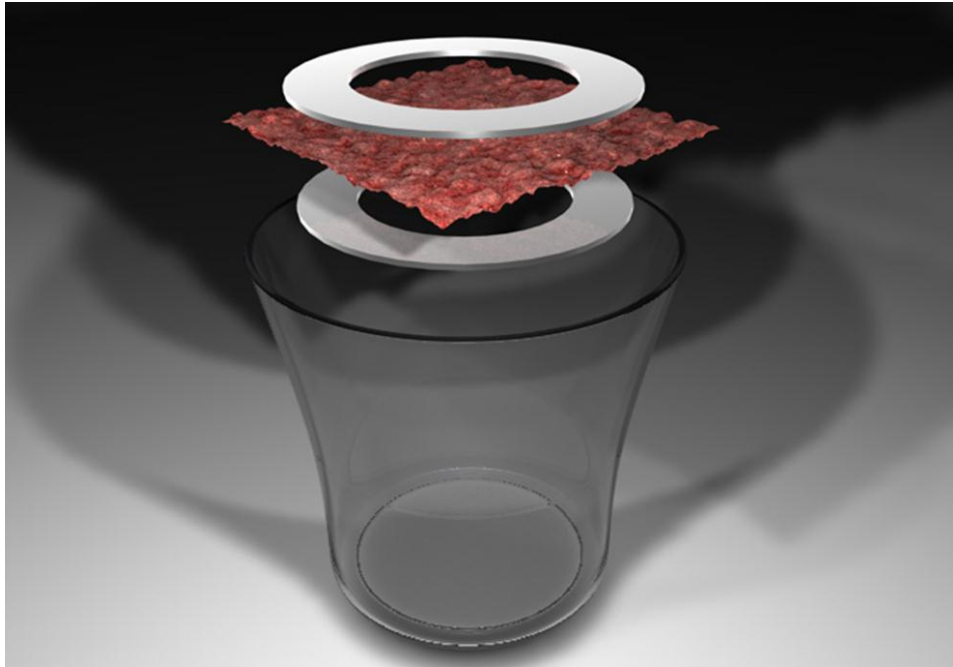
A graphical representation of the EVISC model setup. Figure shows the assembly of the spacer and the intestinal segment into each cell culture insert that is then placed into a well of a 12-well cell culture plate.

Figure 2A
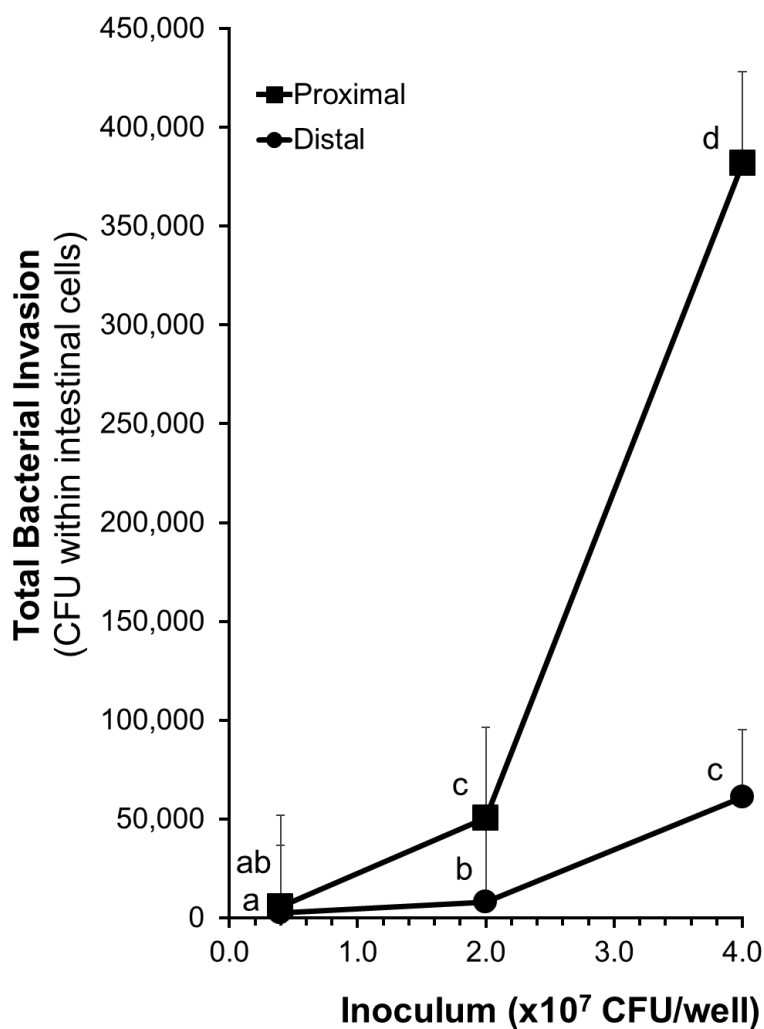
Effects of ileal region (proximal vs. distal ileal segments) and ExPEC inoculum concentration on susceptibility of ileal tissue to invasion by ExPEC. Figures show (A) total invasion and (B) invasivity (Invaded CFU/Inoculum CFU). Means without a common letter differ, $P < 0.05$.

Figure 2B
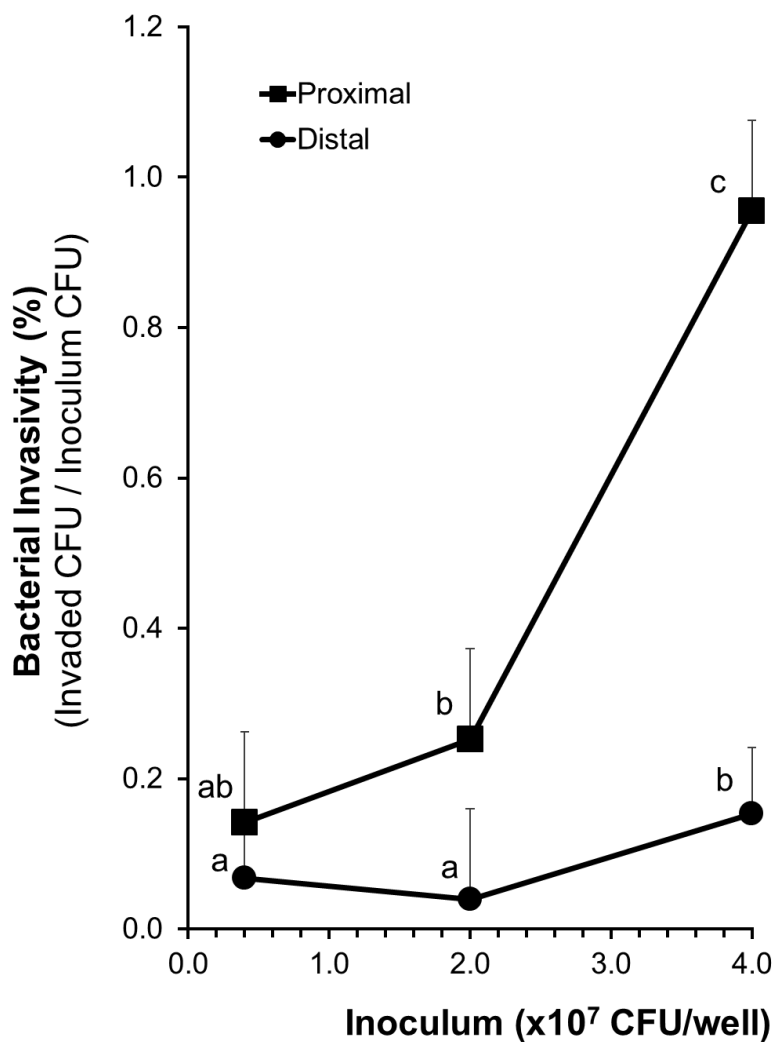
Effects of ileal region (proximal vs. distal ileal segments) and ExPEC inoculum concentration on susceptibility of ileal tissue to invasion by ExPEC. Figures show (A) total invasion and (B) invasivity (Invaded CFU/Inoculum CFU). Means without a common letter differ, $P < 0.05$.

Figure 3
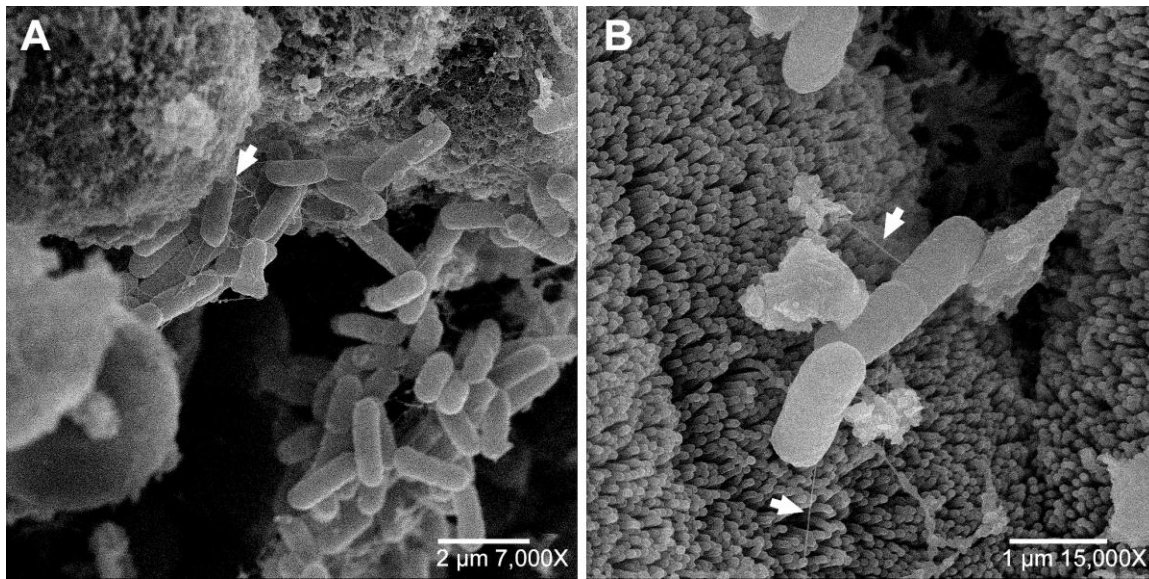
Representative scanning electron photomicrographs of the invasion of ileal epithelial cells by ExPEC. (A) Shows the adhesion of ExPEC to apical surface of cells via surface virulence factor (fimbriae; example indicated by white arrow), (B) Shows ExPEC invasion of the epithelium cells by initiating uptake, a mechanism commonly utilized by microbial pathogens to gain entry into non-phagocytic host cells (37, 38). The observed photomicrographs are identical to what we have previously observed in our studies exploring the invasion of enterocytes (Caco-2 cells) in culture by ExPEC (data not shown).

Figure 4
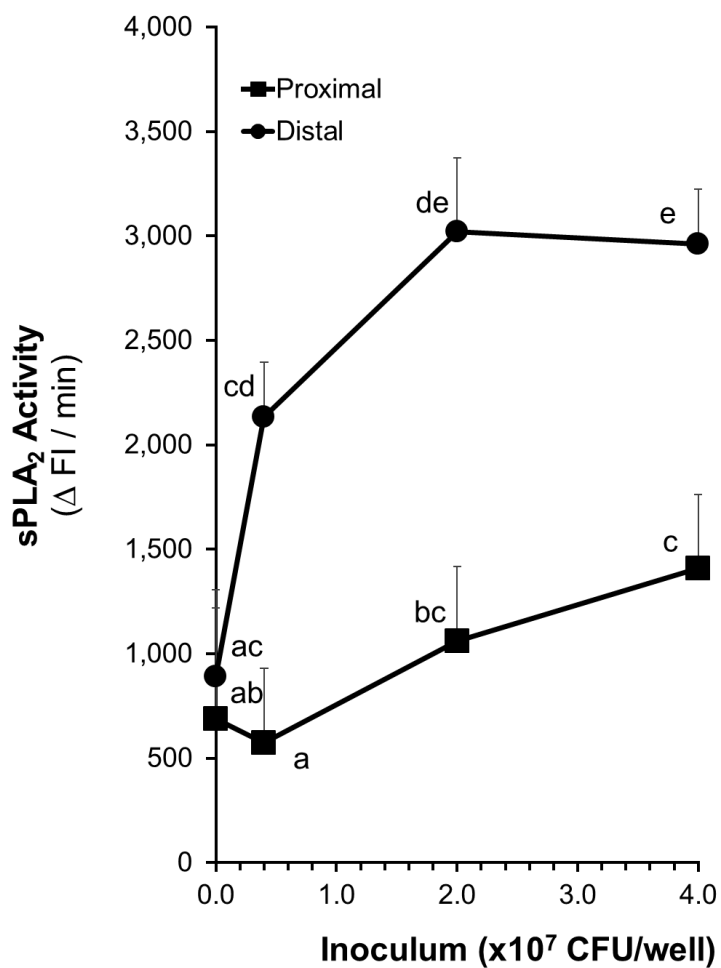
Effects of ileal region (proximal vs. distal ileal segments) and ExPEC inoculum concentration on sPLA$_2$ secretion by mucosal tissue in response to pathogen exposure. sPLA$_2$ activity in the culture media is taken to be a measure of sPLA$_2$ secretion. Means without a common letter differ, $P < 0.05$.

Figure 5A
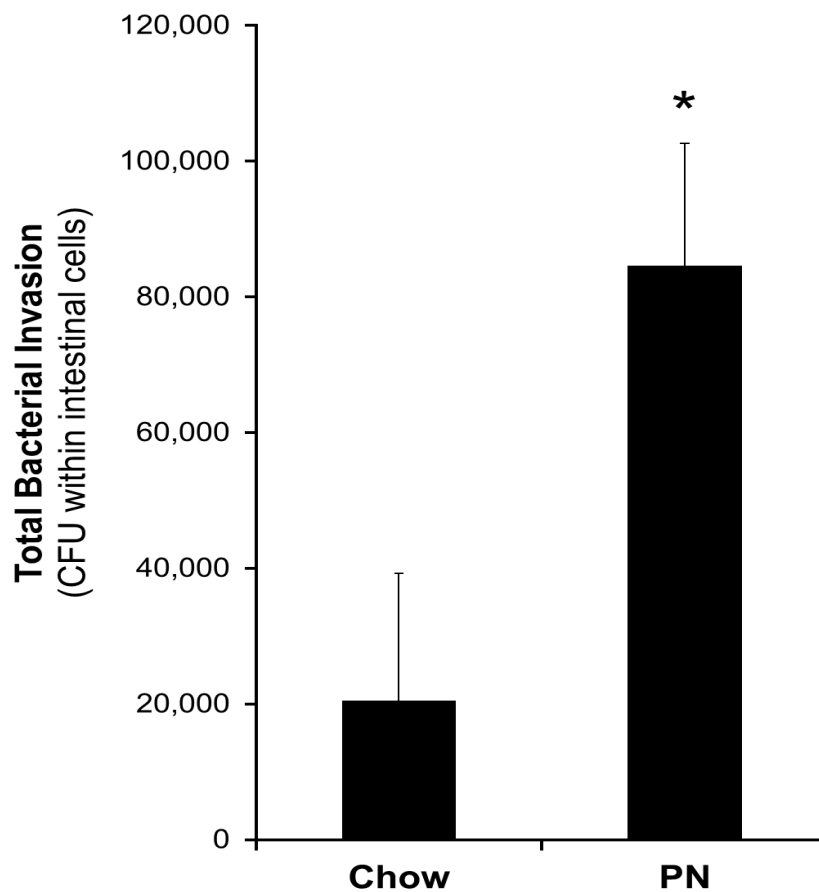
Effect of chow- and PN- feeding on susceptibility of ileal tissue to invasion by ExPEC. Figures show (A) total invasion and (B) invasivity (Invaded CFU/Inoculum CFU). * Denotes statistical difference (P=0.0018) between mucosal tissues from PN- and chow-fed mice.

Figure 5B
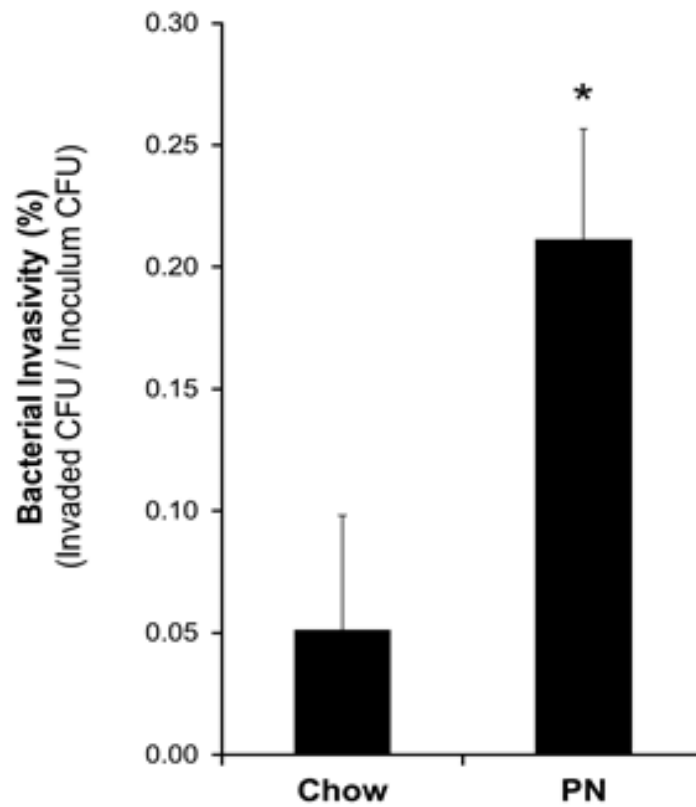
Effect of chow- and PN- feeding on susceptibility of ileal tissue to invasion by ExPEC. Figures show (A) total invasion and (B) invasivity (Invaded CFU/Inoculum CFU). * Denotes statistical difference (P=0.0018) between mucosal tissues from PN- and chow-fed mice.

Figure 6
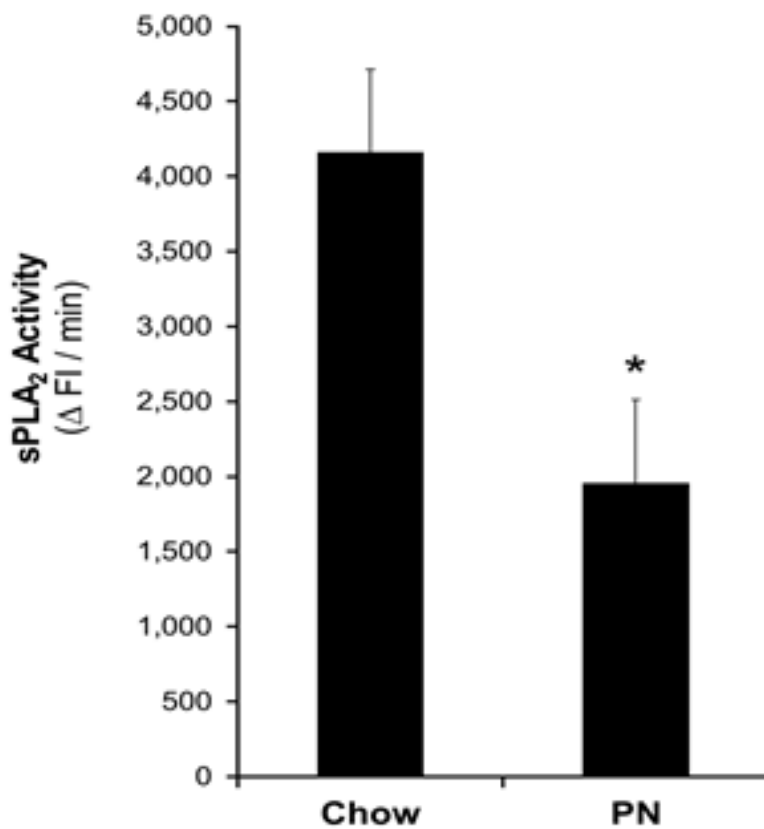
Effect of chow- and PN- feeding on sPLA$_2$ secretion by mucosal tissue in response to pathogen exposure. sPLA$_2$ activity in the culture media is taken to be a measure of sPLA$_2$ secretion. * Denotes statistical difference (P=0.01) between secretions by mucosal tissues from PN- and chow-fed mice.

Chapter 9

IL-25 improves luminal innate immunity during parenteral nutrition.

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ABSTRACT

Background: Parenteral Nutrition (PN) increases infections in critically injured patients. Recently, PN was found to reduce intestine luminal levels of the Paneth cell antimicrobial molecule, secretory phospholipase A_2 ($sPLA_2$), and the Goblet cell glycoprotein, MUC2. These molecules are critical factors in innate mucosal immunity that provide barrier protection. IL-4 and IL-13 regulate $sPLA_2$ and MUC2 production through the IL-13 receptor. Since IL-25 stimulates IL-4 and IL-13 release and since PN reduces luminal $sPLA_2$ and MUC2, we hypothesized that adding IL-25 to PN would restore these innate immune factors and maintain barrier function.

Methods: 2 days after venous cannulation, male ICR mice were randomized to receive Chow (n=12), PN (n=9), or PN + 0.7 μg of exogenous IL-25 (n=11) daily for 5 days. Small intestine wash fluid (SIWF) was collected for analysis of $sPLA_2$ activity, MUC2, and luminal levels of IL-4 and IL-13. Small intestinal tissue was removed for analysis of tissue $sPLA_2$ activity or used immediately in an *ex vivo* intestinal segment culture (EVISC) to assess susceptibility of the tissue segments to *E. coli* enteroinvasion.

Results: PN reduced luminal $sPLA_2$ ($p<0.0001$) and MUC2 ($p<0.002$)compared with chow while the addition of IL-25 to PN increased luminal $sPLA_2$ ($p<0.0001$)and MUC2 ($p<0.02$) compared to PN. Tissue IL-4 and IL-13 decreased with PN compared to chow (IL-4: $p<0.0001$)(IL-13: $p<0.002$), while IL-25 increased both cytokines compared to PN (IL-4: $p<0.03$)(IL-13: $p<0.02$). Tissue levels of $sPLA_2$was significantly decreased in PN compared to Chow, while IL-25 significantly increased tissue $sPLA_2$ levels compared to PN alone. Functionally, more bacteria

invaded the PN treated tissue compared to Chow (p<0.01), and the addition of IL-25 to PN decreased enteroinvasion to Chow levels (p<0.01).

Conclusions: PN impairs innate mucosal immunity by suppressing luminal sPLA$_2$ activity and MUC2 density compared to Chow and increasing bacterial invasion in *ex vivo* tissue. Exogenous IL-25 reverses this dysfunction. IL-25 stimulates Goblet and Paneth cell hypertrophy and increases luminal sPLA$_2$ and MUC2. PN tissue treated with IL-25 was significantly more resistant to bacterial invasion than PN alone, suggesting IL-25 induced effects fortify the barrier defense mechanisms.

INTRODUCTION

Parenteral nutrition (PN) provides nutritional support to patients unable to be fed enterally. Unfortunately, PN is associated with an increased risk of infectious complications [1-4]. Compromise of the intestinal mucosal barrier is believed to be partly responsible for this increased risk [5]. Multiple components of the intestinal mucosal barrier work in concert to prevent bacterial pathogens from attaching to and invading the host tissue [6]. These barrier mechanisms include tight-junction proteins between epithelial cells, mucin glycoproteins secreted by Goblet cells [5, 7], antimicrobial molecules released from Paneth cells [8-11], and the release of immunoglobulin-A (IgA) by the epithelium [12-14]. PN with lack of enteral stimulation impairs multiple components of this barrier function, such as luminal IgA produced by acquired (adaptive) immunity through mechanisms previously reported [15-23]. In addition to impairment of acquired immunity, we recently demonstrated that lack of enteral feeding with PN reduced levels of the Paneth cell antimicrobial molecule, secretory phospholipase A_2 ($sPLA_2$), and the Goblet cell glycoprotein mucin2 (MUC2), within the gut lumen [24].

Innate immunity is regulated by specialized epithelial cells which sustain mucosal defenses by providing a physical, protective barrier and by specialized bioactive molecules. The production and secretion of mucus by Goblet cells plays an integral primary role by providing a physical barrier that expands over and protects the mucosa. Paneth cells produce, store and secrete various antimicrobial peptides which neutralize microbes. Importantly, secreted IgA and Paneth cell antimicrobial peptides localize to mucus layer in high concentrations, which reinforces the mucus layer and limits the exposure of microbes to the epithelium [6, 25].

The Th2 cytokines IL-4 and IL-13 are important mediators for goblet cell mucus production in the intestine [26-28]. IL-13 interacts with the IL-4 receptors (IL-4R) and the IL-13R to promote

production and release of Goblet cell products such as MUC2. Mice develop a lethal colitis in the absence of MUC2 production [29]. Paneth cells produce small antimicrobial proteins including sPLA$_2$, defensins, and lysozymes which are produced and stored in their granules [8, 30-32]. sPLA$_2$ specifically provides antimicrobial activity against Gram-positive bacteria [33-35]. IL-13, which interacts with the IL-13R, is necessary to maintain production of antimicrobial peptides including sPLA$_2$ and various other cryptdins in Paneth cells [28]. IL-13 knockout mice produce lower levels of these antimicrobial products compared to mice with intact IL-13 signaling [28]. These products, in part, comprise the mucosal barrier and help prevent enteroinvasion of pathogenic bacteria into the epithelium.

IL-25 is a powerful cytokine which amplifies Th2 responses [36]. Exogenous administration of IL-25 attenuates colitis in animal models and up-regulates production of the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 [37]. Other groups have demonstrated these cytokines promote Paneth cell and Goblet cell hyperplasia, hypertrophy, and secretion [28], in addition to smooth muscle contraction [38], resulting in what is described as a "weep and sweep" effect on the mucosa that promotes the clearance of pathogens [39]. Since PN reduces the Th2 cytokines IL-4 and IL-13 and the Paneth and Goblet cell products, we hypothesized that adding IL-25 to PN would restore the Th2 response and stimulate innate luminal factors including Goblet Cell MUC2 and Paneth Cell sPLA$_2$, along with restoration of tissue levels of IL-4 and IL-13. We further hypothesized that loss of these products in PN would result in increased susceptibility to bacterial invasion in an *ex vivo* intestinal segment culture (EVISC) and that *in vivo* administration of IL-25 during PN would decrease bacterial invasion.

MATERIALS AND METHODS

Animals

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

Experimental Design

Male ICR mice, ages 6 to 8 weeks, were randomized to Chow with an intravenous catheter (Chow, n = 12), PN (n=12), or PN with 0.7 µg/mouse/day exogenous IL-25 (R&D Systems) delivered IV for 5 days (PN+IL-25, n=12). Animals were anesthetized by intramuscular injection, weighed, and underwent placement of silicon rubber catheter (0.012-inch I.D./0.025-inch O.D.; Helix Medical, Inc., Carpinteria, CA) in the vena cava through the right external jugular vein. The catheter was tunneled subcutaneously and existed at the midpoint of the tail. The animals were housed individually in metabolic cages with wire floors to prevent coprophagia and bedding ingestion and partially immobilized by tail restraint to protect the catheter during infusion. This technique has proven to be an acceptable method of nutritional support and does not produce physical or biochemical evidence of stress. The catherized mice were connected to infusion pumps and received saline (0.9%) at 4 mL/day and *ad libitum* chow (Agway Inc., Syracuse, NY) and water during 48 hours of recovery After 48 hours, Chow mice

continued to receive 0.9% saline at 4 mL/day as well as *ad libitum* chow and water. PN animals

received PN solution at rates 4 mL/d (day 1), 7 mL/d (day 2) and 10 mL/d (day 3 to 5) because a

graded infusion period was demonstrated to be necessary for the mice to adapt to the glucose and

fluid loads . The PN solution contained 6.0% amino acids, 35.6% dextrose, electrolytes, and

multivitamins, containing 1440 kcal/L and a non-protein calories/ nitrogen ratio of 128:1. These

values were calculated to meet the nutrient requirements of mice weighting 25 to 30 g.

After 5 days of feeding (7 days post-catheterization), mice were anesthetized by

intraperitoneal injection of ketamine (100 mg/kg) and acepromazine (10 mg/kg), and

exsanguinated via left axillary artery transection. The small intestine was removed and the lumen

rinsed with 20 mL Hanks Balanced Saline Solution (HBSS, Bio Whittaker, Walkersville, MD).

The luminal fluid was centrifuged at 2,000 x g for 10 min and supernate aliquots were frozen at

-80°C for $sPLA_2$ activity, MUC2 densitometry, and luminal IL-4 and IL-13 levels. Tissue

samples were taken by removing ileal segments (3 cm) excluding Peyer's patches. These

samples were frozen in liquid N_2 and stored at -80°C until processing or used immediately in

 the *ex vivo* intestinal segment culture to determine bacterial enteroinvasion. .

Continuous Fluorescent Assay for sPLA$_2$ Activity

Fluorescent assay for $sPLA_2$ activity was performed as described previously by *Tsao et al*, with

some modification to substrate preparation. This assay uses a specific probe, Bis-BODipy FL,

which is designed to fluoresce when the Sn2 position of the phospholipid glyceraldehyde

backbone is cleaved. This method was established as a high throughput method to rapidly

analyze $sPLA_2$ activity, and we have reliably utilized this technique to measure $sPLA_2$ activity in

the small intestinal tissue and luminal fluid. [24]. After the reactions reached equilibrium

temperature, the reaction curve was fit to a second-order polynomial equation and the first-degree coefficient was taken as the initial rate of reaction (expressed as change in Fluorescence (FL)/min/uL sample). Blank wells containing only substrate and buffer were used to find coefficient rates determined as background activity.

Western Blot for MUC2 in the Small Intestinal Wash Fluid

 5 uL of tissue culture media was denatured and separated at 95 ^{o}C for 10 min with sodium dodecylsulfate and β-mercaptoethanol and separated in a 4-15 % polyacrylamide gel (Ready Gel, Bio-Rad Laboratories, Hercules, CA) by electrophoresis at 150 V for 65 min. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the standard transfer buffer (Tris-glycine buffer plus 20% methanol) at 80 V for 60 min. Membranes were blocked with 5 % nonfat dry milk prepared in TBS-Tween (Tris-buffered saline with 0.5% Tween-20) for 1 hour with continuous stirring. The membranes were incubated with the primary antibody, mouse anti-human MUC2 (ab-11197, Abcam Inc, Cambridge, MA) diluted 1:2500 overnight at 4 °C with constant rocking. Membranes were washed and incubated with stabilized goat anti-mouse IgG-HRP conjugate (sc-2005, Santa Cruz Biotechnology, CA) diluted 1:20,000 for 1 hour at room temperature with constant agitation. After washing with TBS-tween, membranes were incubated with HRP substrate (Super Signal West Femto maximum sensitivity substrate; Pierce, Rockford, IL) for 5 min and bands were detected using photographic film. Densitometric analysis of molecular weight bands at 260kDa and 500 kDa were used to measure MUC2 concentration.

Tissue and Luminal Cytokine Quantitative Analysis

Concentrations of IL-4 and IL-13 were measured in the small intestinal tissue or in the small intestinal wash fluid (SIWF) using a solid phase sandwich ELISA (BD Biosciences, San Diego, CA) as previously described [40]. The mass amounts were determined by plotting sample absorbance values on a 4-parameter logistic fit standard curve, as calculated with SOFTmax PRO software (Molecular Devices) and were normalized to total protein content.

Bacterial Preparation

Escherichia coli 5011-Lux containing ampicillin resistance were grown in 40 mL LB broth for 48 hours at 37 ℃ under 5% CO_2, then a surface sample was passed to a new 40 mL LB broth and grown for 24 hours at 37ºC under 5 % CO_2. The surface sample is used since bacteria growing near the culture surface express virulence. Bacteria were spun down at 1780 x g for 11 min, the supernate was poured off, and the pellet was re-suspended in 40 mL LB to wash twice, and then re-suspended in 1 mL DPBS at 4 ℃ as a bacteria stock solution. A 1:100 dilution of the bacterial stock solution was made and read on a spectrophotometer (machine, company, city, state) at 450 nm wavelength. Bacterial concentrations were adjusted based on growth curves previously established.

Ex Vivo Intestinal Segment Culture

Intestinal Segments were placed on a sterile surface covered in a light layer of RPMI and carefully opened apical side up. Tissue glue (Dermabond, Ethicon, Cornelia, GA) was lightly covered on one side of a plastic tissue disc and lowered over the intestinal segment. Tissue discs were manufactured from polystyrene with a 9 mm diameter and 6 mm internal aperture. Once the tissue glue set (approximately 10 seconds) the tissue disc and intestinal segment were turned

over. A second tissue disc was lightly covered with tissue glue and placed on the serosal side of the intestinal segment. Once the second tissue disc was adhered, a light layer of tissue glue was applied to the bottom of the serosal disc and the intestinal segment sandwiched between tissue discs were lower into a cell culture insert (Cat 3292, 3.0 µM pore, 12 well format, BD bioscience, NJ). Gentle pressure was applied to ensure adherence of the bottom tissue disc to the cell culture insert. Schematic of tissue discs, intestinal segment, and cell culture insert are shown in *Image 1*. Cell culture inserts were placed into 12 well plates prefilled with 1 mL RPMI+Ampicillin per well.

400 µL of respective bacterial inoculums ($1x10^8$ Colony Forming Units (CFU)/mL) in RPMI+Ampicillin was placed in appropriate wells for 1 hour at 37ºC. Then the bacterial inoculum was collected, centrifuged at 14,000g for 2 minutes to pellet bacteria, and the supernate stored for analysis of mucosal secretions. The wells were rinsed by gently adding and removing 500 µL of DPBS 3 times. Then 600 µL RPMI+Gentamicin was added to each well for 1 hour at 37ºC. This was done to kill any remaining bacteria in the well or adhered to the mucosal surface. Then the RPMI+Gentamicin were removed and the wash step repeated. Then 500 µL of 0.1% Triton-X in PBS was added to each well. The 12 well plates were placed on an orbital shaker and agitated (175 rpm; New Brunswick Scientific Classic Series C1 Shaker) for 30 minutes at room temperature. Serial dilutions (10^1-10^7) of the cell lysate made in DPBS and plated on LB+Ampicillin agar plates which were gorwn for 18 hours at 37ºC. Enteroinvasion was assessed by counting CFUs. The RPMI+Ampicillin added to the 12 well plate was also plated to detect cell culture insert leaks. If leaks were detected from the apical to serosal side, the well was discarded from the experiment.

Statistical analysis

The data are expressed as means ± standard error of the mean. Statistical significance was determined using ANOVA with Fisher's protected least significant difference *post hoc test* or Student's *t*-test. Differences were considered to be statistically significant at *p<0.05*. All statistical calculations were performed with StatView (Abacus Concepts, Berkeley, CA).

RESULTS

Analysis of Intestinal Fluid sPLA$_2$

The sPLA$_2$ activity (FL/min/uL) within the small intestinal wash fluid was significantly lower in PN compared to Chow (1895 ± 418 vs. 7811 ± 467, p < 0.0001). The addition of IL-25 to PN mice significantly increased sPLA$_2$ activity compared to PN alone (5954 ± 824 vs. 1895 ± 418, p < 0.0001); sPLA$_2$ was still significantly lower in IL-25 than Chow fed mice (5954 ± 824 vs. 7811 ± 467, p = 0.03) (Figure 1).

Analysis of Intestinal Fluid MUC2

The luminal MUC2 (relative density) within the small intestinal wash fluid was significantly depressed in PN mice compared to Chow (50366 ± 4059 vs. 75912 ± 6181, p = 0.001). PN supplementation with IL-25 significantly increased the relative luminal level of MUC2 compared to PN (65448 ± 4625 vs. 50366 ± 4059, p = 0.02). There were no significant differences between chow and IL-25 (75912 ± 6181 vs. 65448 ± 4625, p = 0.16) (Figure 2).

Analysis of Tissue IL-4 and IL-13 Levels

PN significantly reduced tissue levels of IL-13 (pg/mg) compared to chow (PN: 8.1 ± 1.0 vs Chow: 15.3 ± 1.8, p = 0.002) (Figure 3A). The addition of IL-25 to PN significantly increased IL-13 tissue levels compared to PN alone (PN+ IL-25: 13.9 ± 1.6 vs PN: 8.1 ± 1.0, p = 0.005) and the IL-13 levels were simila rto Chow levels (PN+ IL-25: 15.3 ± 1.8 vs Chow: 13.9 ± 1.6, p = 0.5). Consistent with our previous studies, PN significantly decreased the tissue levels of IL-4 compared to chow fed mice (PN: 22.1 ± 0.9 pg/mg protein vs Chow: 34.5 ± 2.7 pg/mg protein, p = 0.0003) (Figure 3B). The addition of IL-25 to PN significantly increased tissue IL-4 compared to PN alone (PN+ IL-25: 28.4 ± 1.8 pg/mg protein vs PN: 22.1 ± 0.9 pg/mg protein, p = 0.03), the IL-4 levels remained lower that Chow (PN+ IL-25: 28.4 ± 1.8 pg/mg protein vs Chow: 34.5 ± 2.7 pg/mg protein, $p < 0.05$).

Analysis of tissue levels of IL-4 and IL-13

PN significantly decreased the luminal levels of IL-4 and IL-13 (pg/mL) compared to Chow (IL-4: 119.3 ± 8.1 vs. 174.8 ± 9.1, p = 0.16) (IL-13: 3.9 ± 0.4 vs. 5.8 ± 0.9, $p < 0.05$). The addition of IL-25 to PN significantly increased IL-4 compared to PN alone (146.4 ± 8.7 vs119.3 ± 8.1, p = 0.02) but was still significantly depressed compared to Chow (146.4 ± 8.7 vs. 174.8 ± 9.1, p = 0.04). PN + IL-25 significantly increased the luminal levels of IL-13 compared to PN (5.1 ± 0.4 vs. 3.9 ± 0.4, $p < 0.05$) and the IL-13 was statistically similar to the Chow group (5.1 ± 0.4 vs. 5.8 ± 0.9, p = 0.2) (Figure 4A and 4B).

Analysis of Tissue sPLA$_2$

sPLA$_2$ activity (Fl/min/uL) within the ileum tissue was significantly reduced in PN compared to Chow (1895 ± 418 vs. 7811 ± 467, $p < 0.0001$). The addition of IL-25 to PN significantly

increased tissue sPLA$_2$ activity compared to PN (5954 ± 845 vs. 1895 ± 418, p < 0.0001) but IL-25 was still significantly decreased compared to Chow (5954 ± 845 vs. 7811 ± 467, p = 0.03) (Figure 5).

Bacterial Recovery after EVISC

Ileum segments from PN fed animals were significantly more susceptible to bacterial enteroinvasion (CFUs/segment) compared to Chow segments (20450 ± 4290 vs 84851± 24671, p < 0.01). The addition of IL-25 to PN significantly decreased bacterial enteroinvasion compared to PN alone (13497 ± 4400 vs 84851± 24671 CFUs, p < 0.01) and was statistically similar to bacterial invasion in chow fed mice.

DISCUSSION

Parenteral Nutrition (PN) prevents progressive malnutrition in patients with contraindications to enteral nutrition. Unfortunately, lack of enteral feeding is associated with an increased risk of infectious complications, a risk suspected to be due in-part to alterations in the intestinal mucosal barrier. The mucosa is protected by both adaptive (acquired) immunity and innate immunity. Adaptive immunity functions to produce and release IgA at mucosal surfaces. Innate immunity of the mucosal barrier includes the release of antimicrobial molecules from Paneth cells and secreted mucin glycoproteins by goblet cells that cover the epithelial surface. Under normal circumstances these and other mechanisms successfully defend surface area of roughly 300 meters^2 against dietary and environmental antigens and enormous numbers of bacteria [5]. We previously demonstrated that PN feeding suppresses luminal levels of IgA and the Paneth cell

antimicrobial compound, sPLA$_2$ [24, 41, 42]. In addition, we observed PN with lack of enteral stimulation reduces the goblet cell derived mucin layer, which is largely composed of the MUC2 glycoprotein [5, 7]. This work investigated whether deleterious changes in innate immunity following PN can be corrected via administration of the Th2 cytokine IL-25 during PN feeding. The Th2 cytokines IL-4 and IL-13 are mediators of goblet cells and mucus production in the intestine [26-28, 43]. IL-13 interacts with the IL-4 receptors (IL-4R) and the IL-13R to promote production and release of the glycoprotein MUC2. IL-13 is also a mediator of Paneth cells, where its interactions with IL-13R is necessary to stimulate production of antimicrobial peptides including sPLA$_2$ and cryptdins. IL-13 knockout mice produce low levels of these antimicrobial products [28]. IL-25 is a powerful cytokine which amplifies Th2 responses and appears to be produced and by the epithelium itself [36]. Recent experiments demonstrate exogenous administration of IL-25 attenuates colitis in animal models and up-regulates production of the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 [37]. Collectively, these studies show the Th2 profile promotes Paneth and Goblet cell hyperplasia, hypertrophy, and secretion, in addition to smooth muscle contraction [38, 39]. These effects result in what is a "weep and sweep" effect on the mucosa that promotes the clearance of pathogens [39].

We studied the effects of PN and PN + IL-25 on the innate immune products released by two types of secreting epithelial cells, Paneth Cells and goblet cells, which provide innate defenses for the intestinal mucosa. Goblet cells produce mucin glyoproteins that provide a physical barrier between organisms within the lumen and the epithelial cell layer. The most abundant mucin secreted into the small intestine is MUC2. This large glycoprotein provides a visoelastic surface over which the intestinal contents pass. Paneth cells reside at the base of the intestinal crypts where they produce, store and secrete an array of constitutively expressed antimicrobial

products, including $sPLA_2$, lysozyme, and the defensins, that provide a chemical defense against luminal microorganisms [31, 32, 44]. $sPLA_2$ attacks Gram positive bacterial cell membranes where it catalytically cleaves phospholipids, inducing membrane disruption and lysis [44]. Interestingly, the many Paneth cell antimicrobial molecules, including $sPLA_2$, are cationic and concentrate within the highly anionic MUC2 layer. Together, these molecules form an immunological barrier preventing attachment of bacteria and compromise of the mucosal epithelium. The importance of these mechanisms is demonstrated in mice lacking either Paneth or goblet cell products. These mice are impaired in clearing infectious organisms and more easily succumb to epithelial inflammation and colitis that may be lethal [29].

This study was consistent with our previous work which demonstrated PN significantly reduced luminal levels of the goblet cell product, MUC2, and the Paneth cell product, $sPLA_2$ [24]. PN also decreased the expression of the Paneth cell product $sPLA_2$ in tissue homogentates, suggesting Paneth cell production is affected. Since Paneth and goblet cell products are stimulated in-part through the Th2 cytokines IL-4 and IL-13, we also measured these molecules in the tissue and showed their levels were significantly lower following PN, consistent with our previous reports. This study also showed a reduction in the luminal expression of the Th2 cytokines, IL-4 and IL-13. Interestingly, the luminal cytokine levels mirrored the tissue levels, suggesting a link between the two compartments. Other groups have concluded luminal cytokines may provide paracrine signals to the epithelium under inflammatory conditions [45-47]. To our knowledge, this is the first report of changes to luminal cytokines following PN feeding, and we are currently investigating the implications and functions of these and other luminal cytokines in maintenance of immune function.

To determine the stimulatory role of Th2 cytokines upon tissue and luminal levels of these cell products, we exogenously administered the Th2 amplifying cytokine, IL-25, during PN. The administration of IL-25 with PN significantly increased tissue sPLA$_2$ levels, implicating a stimulatory effect on Paneth cells. IL-25 administration to PN also significantly increased the tissue levels of Th2 cytokines IL-4 and IL-13 compared with PN alone. Finally, the addition of IL-25 to PN also significantly increased luminal levels of sPLA$_2$ and MUC2 compared to PN alone. This suggests Th2 cytokines are important for the production of these Paneth and goblet cell products. Consistent with the tissue cytokines, luminal IL-4 and IL-13 were also higher in the wash fluid after exogenous IL-25 treatment of PN, further supporting a possible role for luminal cytokines.

To determine if IL-25 administration to PN had functional consequences on barrier function, we challenged the small bowel tissue using an *ex vivo* intestinal segment culture model designed to quantify bacterial enteroinvasion. Consistent with suppressed parameters of the mucosal barrier following PN feeding, such as the loss of sPLA$_2$ and MUC2, small bowel segments form PN animals had significantly greater enteroinvasion compared to chow. Strikingly, compared with PN alone, the addition of exogenous IL-25 to PN significantly decreased enteroinvasion to levels observed in chow segments. Since IL-25 increased luminal levels of sPLA$_2$ and MUC2, it is possible these secreted molecules are protective in the *ex vivo* model. Further studies are underway to investigate this possibility and further elucidate protective mechanisms of barrier function. One limitation of this study is we used sPLA$_2$ as a surrogate marker for Paneth cell production in tissue and lumen, since sPLA$_2$ is a constitutively expressed molecule. However, there are other antimicrobial compounds produced and secreted by Paneth cells, including

Lysozyme, RegIII, Ang-4, and the cryptidins in addition to sPLA$_2$. Additional work is underway to investigate these compounds.

In summary, this study supports the hypothesis that PN with the lack of enteral stimulation induces dysfunctional mucosal barrier responses, as measured through the loss of MUC2, sPLA$_2$, and increased susceptibility to *Escherichia coli* enteroinvasion. Further, this study demonstrates that the Th2 amplifying cytokine, IL-25, stimulates production of the cytokines IL-4 and IL-13 and restores the luminal concentrations of sPLA$_2$ and MUC2, changes which coincide with restored barrier function in our *ex vivo* enteroinvasion challenge. Further work is underway to determine contributions of MUC2, sPLA$_2$, and other Paneth cell antimicrobials molecules in protection of the mucosal barrier.

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Figure 1
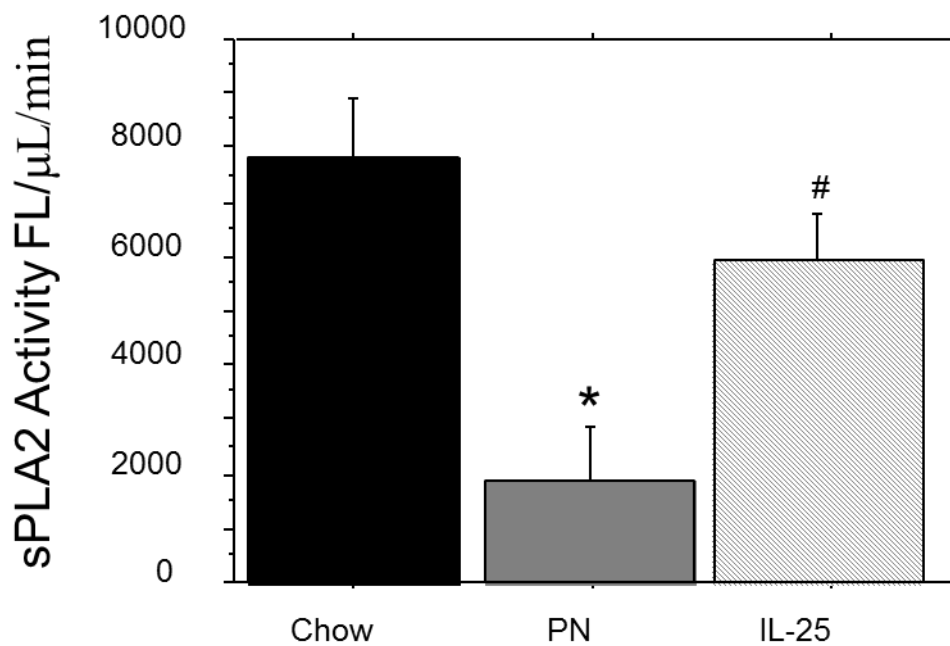
sPLA$_2$ activity from the Small intestinal wash fluid (SIWF). Parenteral nutrition (PN) significantly decreased the luminal levels of sPLA$_2$ compared to chow. IL-25 significantly increased sPLA$_2$ activity similar to chow levels. Data are represented as mean ± SEM. *p < 0.0001 vs Chow and IL-25. #p = 0.03 vs Chow.

Figure 2.
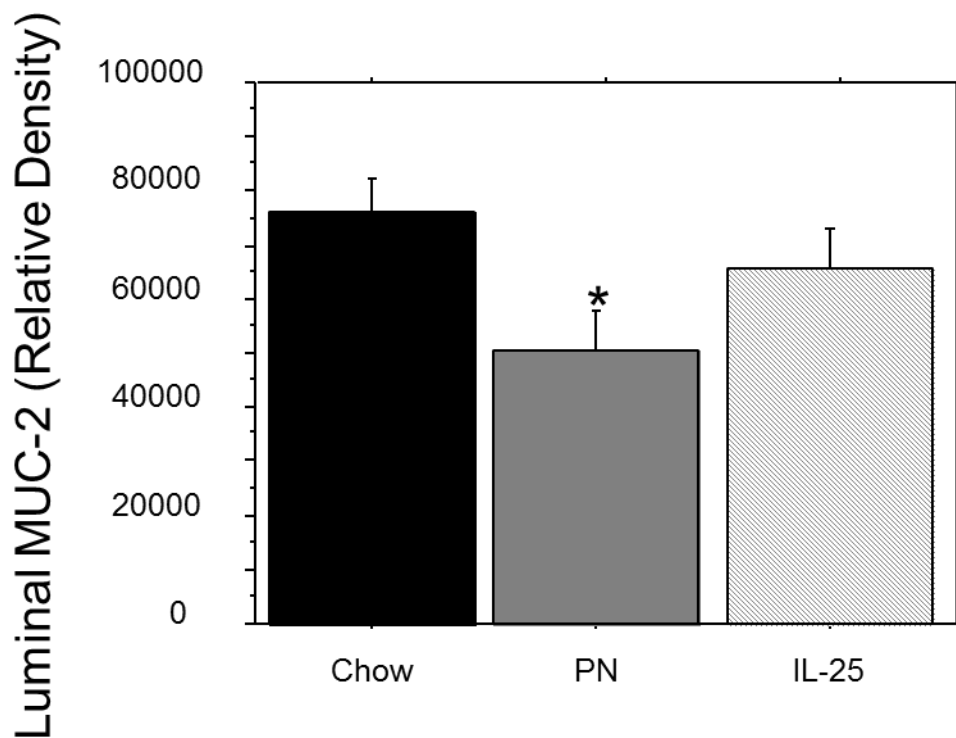
Densitometric analysis of MUC2 in the Small intestinal wash fluid (SIWF). Intravenous parenteral nutrition (PN) significantly decreased the luminal density of MUC2 compared to PN. IL-25 stimulated PN significantly increased luminal MUC2 levels compared to PN alone. Data are presented as mean ± SEM. *p < 0.02 vs Chow and IL-25.

Figure 3
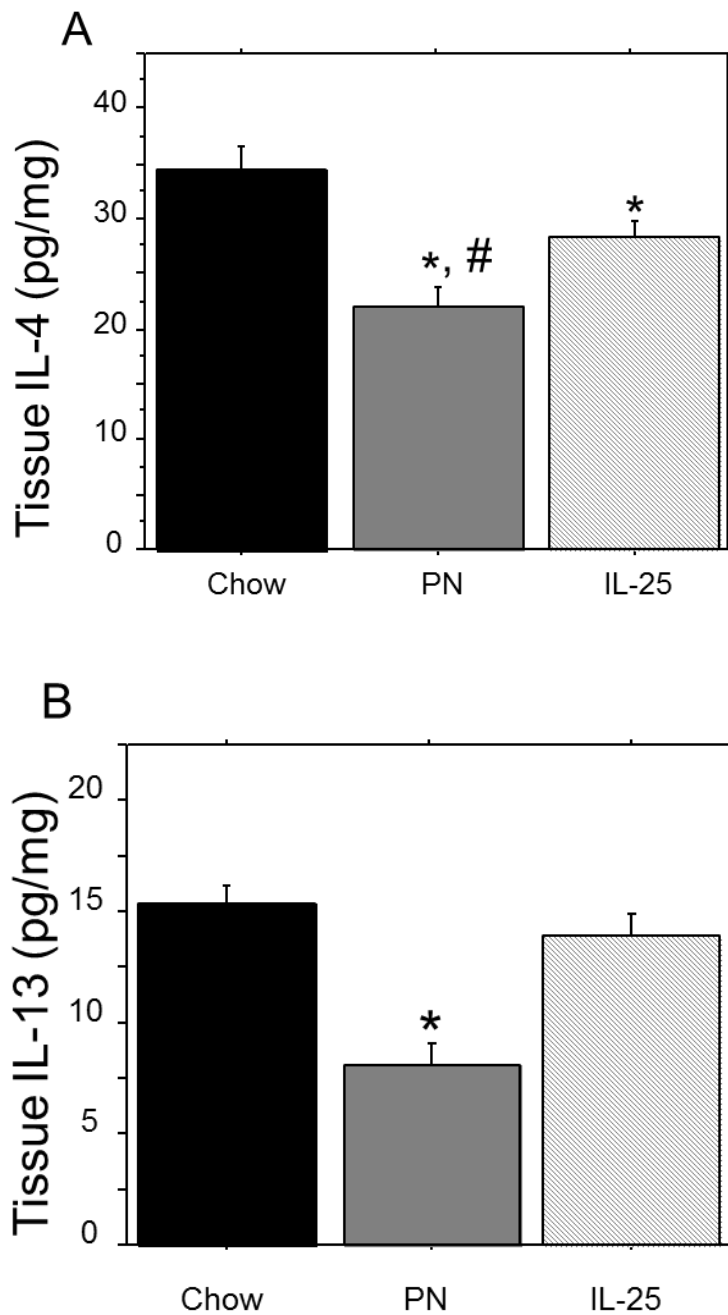
Tissue levels of IL-4 and IL-13 after exogenous IL-25. (A) IL-4 was significantly depressed in PN compared to Chow and IL-25. The addition of IL-25 to PN significantly increased IL-4 compared to PN but failed to restore levels back to chow levels. (B) IL-13 was significantly decreased in PN compared to Chow and IL-25. The addition of IL-25 to PN restored IL-13 to Chow levels. . Data are presented as mean ± SEM. *p < 0.005 vs Chow. #p < 0.03 vs IL-25.

Figure 4
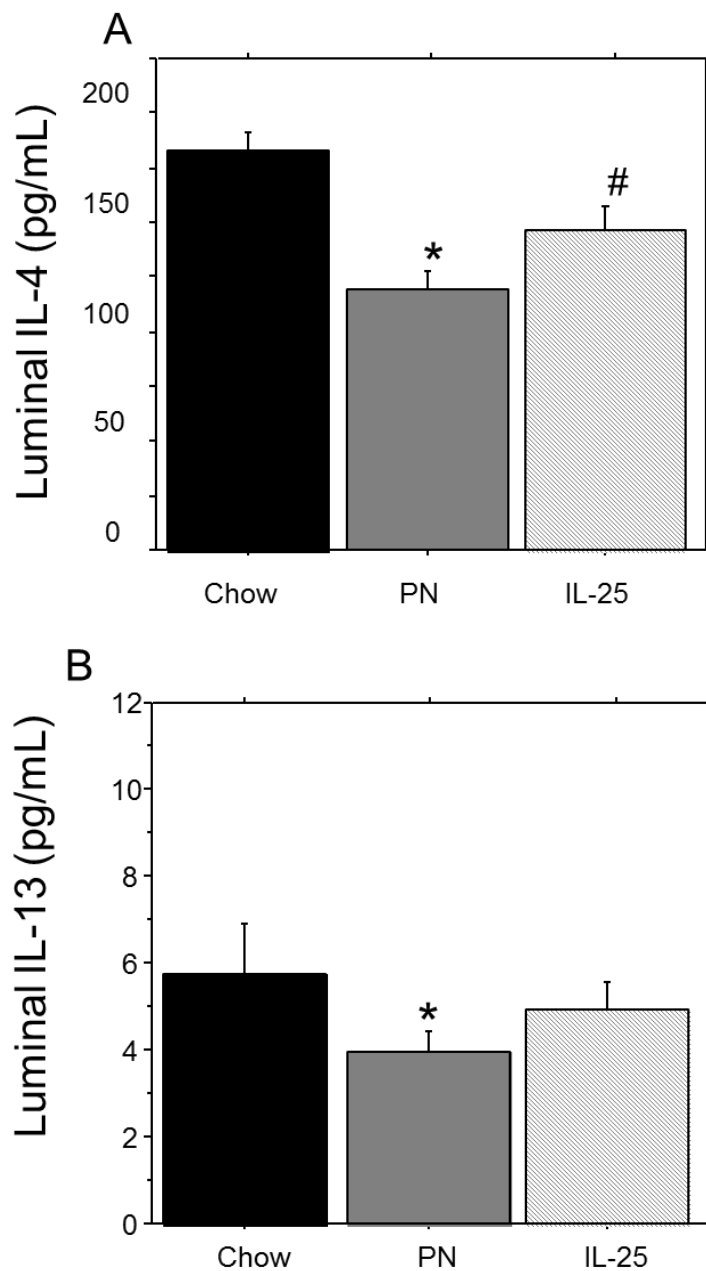
IL-4 concentration in small intestinal wash fluid (SIWF). (A) Parenteral nutrition (PN) significantly decreased the expression of IL-4 compared to Chow. The addition of IL-25 to PN animals significantly increased IL-4 compared to PN alone. (B) IL-13 concentration in the small intestinal wash fluid (SIWF). Parenteral nutrition (PN) significantly suppressed luminal levels of IL-13 compared to Chow. The addition of IL-25 to PN significantly increased the IL-13 similar to chow levels. Data are presented as mean ± SEM. *p < 0.003 vs Chow.

Figure 5
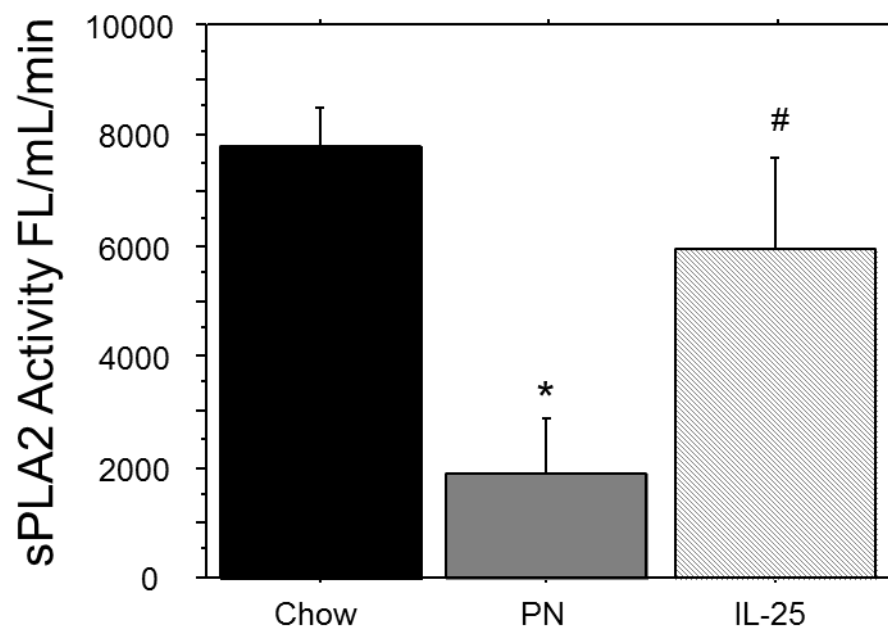
sPLA$_2$ activity from Ileum Tissue. Parenteral nutrition significantly decreased the tissue levels of sPLA$_2$ compared to Chow. IL-25 significantly increased the tissue levels of sPLA2 compared to PN, but the levels were still depressed compared to chow alone. Data are represented as mean ± SEM. *p < 0.05 vs Chow. #p < 0.05 vs PN.

Figure 6.
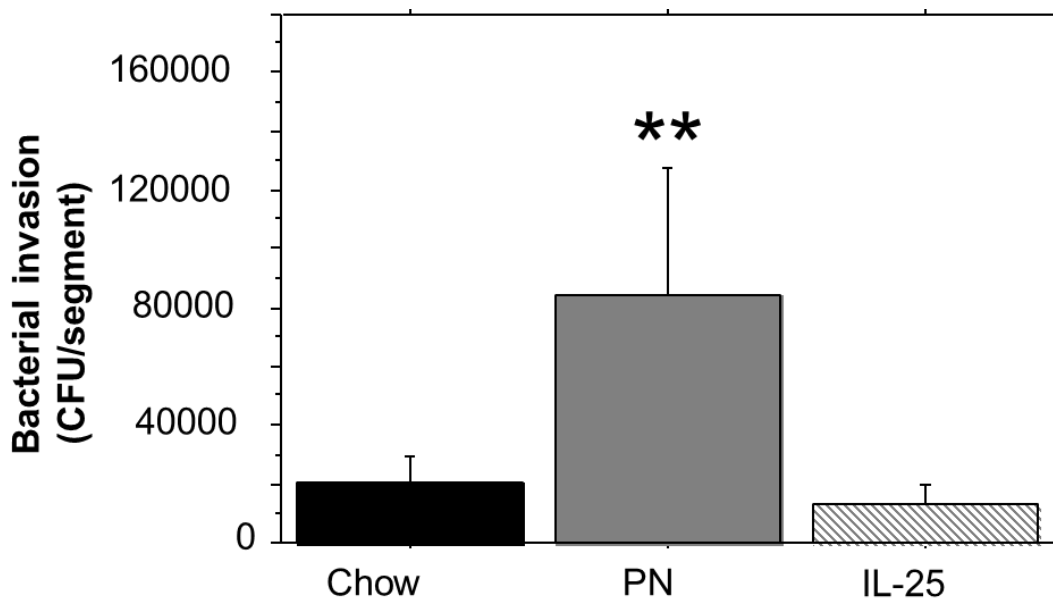
Recovered CFU's after ex vivo intestinal segement culture (EVISC). Parenteral nutrition significantly increased the recovered CFU's compared to Chow. IL-25 significantly decreased the recovered CFU's compared to PN and was statistically similar to CFU's recovered from Chow. Data are represented as mean ± SEM. *p < 0.05 vs Chow. #p < 0.05 vs PN.

Chapter 10

Summary and Future Directions

This thesis explored on the effect of decreased enteral intake upon the mucosal barrier

function in the small intestine. The core hypothesis was that mucosal barrier function of the

small intestine is decreased during reduced enteral intake and that maintenance of barrier

function with therapeutic approaches may be protective to the intestinal mucosa. The first

specific focus was to investigate changes to the Paneth cell antimicrobial compound $sPLA_2$

following PN and EN. As a result, we reported for the first time reduced expression of tissue and

luminal $sPLA_2$ following EN or PN and reduced bactericidal activity following PN that was, in-

part, attributed to $sPLA_2$ activity. The second specific focus was to study the effects of elemental

EN upon mucosal barrier function and investigate the effect of PAC supplementation to

elemental EN upon mucosal barrier function. As a result, we reported changes that occur to the

mucous layer of the intestinal epithelium following elemental EN, including decreased goblet

cell mucous, reduced GALT parameters including less luminal sIgA, and provide preliminary

evidence for the loss of microbiome diversity as well as tissue and luminal levels of $sPLA_2$.

Consistent with the hypothesis, the addition of PACs at physiologic levels maintained each of

these aspects of barrier function compared with elemental EN feeding alone. Protective cytokine

profiles in the small bowel epithelium are generally characterized by increased Th2 levels;

however, these cytokines are decreased with PN and EN. Accordingly, the third focus of this

dissertation was to investigate the effect of administering a Th2 stimulating cytokine, IL-25,

intravenously during PN feeding to explore the effect upon luminal levels of sIgA, goblet cell

mucous, and Paneth cell antimicrobial products in the intestinal lumen. Consistent with the

hypothesis, we observed maintained levels of each of the mucosal barrier parameters with the

addition of IL-25 to PN compared with PN alone. The final focus of this dissertation was to

model the small intestine *ex vivo* for functional investigation of mucosal epithelium and an

enteroinvasive pathogenic strain of *Escherichia coli*. Accordingly, we developed an *ex vivo* intestinal segment culture (EVISC). The EVISC method was employed to explore the infectious susceptibility of ileum tissue following *in vivo* animal feeding trails, specifically the effect of PN and PN+IL-25. Consistent with our hypothesis, we observed PN leads to an increased susceptibility of epithelial enteroinvasion by pathogenic bacteria compared with control animals and that this was prevented with the addition of IL-25 to PN. Together, these data highlight important alterations that occur to the intestinal mucosal barrier following PN and EN, especially in regard to specialized secreting epithelial cells, and lay the ground work for the investigation potential therapeutics during EN and PN that may reduce the risk of infectious susceptibility.

Since the alterations in mucosal barrier function described in this dissertation may increase the host's risk of infectious susceptibility, future directions include further investigation of the alterations that occur in microbiome composition following EN or PN feeding and how these alterations influence and are influenced by the mucosal barrier. The secretion of goblet cell glycoproteins serves as an important fuel source for commensal bacterial populations and is also the site of localization for antimicrobial Paneth cell products and sIgA, therefore proinflammatory shifts in microbiome composition may be expected following PN or EN. The effect of adding exogenous stimulants, such as IL-25, that maintains mucosal barrier secretions may prevent or delay proinflammatory shifts in the microbiome that could aid in maintaining other aspects of immunity, such as GALT function. Similarly, the alterations in microbiome composition following elemental EN feeding that are prevented with PAC supplementation to elemental EN should be explored deeper with 16S sequencing techniques, similar to those used in Appendix 1 that follows, to build upon the preliminary evidence in Chapter 6 of this dissertation.

APPENDIX 1

Microbiome Analysis and Bacterial Enteroinvasion in a Murine Model

of Hirschsprung's Disease

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ABSTRACT

Background: Hirschsprung's disease (HSCR) is characterized by aganglionosis of the distal colon which often manifests as a functional obstruction and can cause severe life-threatening colitis, Hirschsprungs-associated enterocolitis (HAEC). Despite surgical resection of the aganlionic segment, up to 40% of treated patients continue to suffer from HAEC. Investigations have investigated microbiome changes during HAEC.This study focused on changes in host defense and concurrent microbiome alterations in a murine model of HSCR that develop lethal colitis by post-natal 28 (P28) days of age.

Materials and Methods: Conditional mutagenesis was employed to delete *EdnrB*, which is required for NCC migration, in the neural crest: *Wnt1-Cre$^{+/-}$R26R$^{YFP/+}$Ednrb$^{flex3/flex3}$* (*EdnrB* -null) and *Wnt1-Cre$^{+/-}$R26R$^{YFP/+}$Ednrb$^{flex3/+}$* (*EdnrB*-het). Littermates from both groups were sacrificed at P16-18 and P21-24. DNA was isolated from cecal stool samples and Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) employed for microbiome analysis (n=10-15/group/time point). Small bowel tissue was taken for immunohistochemistry of secretory phospholipase A$_2$ (sPLA$_2$), an innate immune antimicrobial protein. Small intestinal wash fluid was collected for measurement of sPLA$_2$ activity by fluorescent assay. A novel *ex vitro* organ culture system was used to measure enteroinvasion of *Escherichia coli* into segmental ileal explants from P21-24 animals.

Results: At the genus level, *EdnrB*-het and –null animals displayed similar flora at P16-18. By P21-24, *EdnrB*-het animals demonstrated an increase in Lactobacillaceae and a decrease in Bacteroidaceae and Clostreidiaceae over time. *Ednrb*-null animals demonstrated reciprocal changes (p<0.001, p=0.001, p=0.003). At p16-18, sPLA2 tissue expression was equal. By P21-

24 tissue sPLA2 expression and luminal activity was decreased (p=0.009). Functionally, *ex vitro* enteroinvasion of segments with E. coli demonstrated *EdnrB* -nulls were significantly more susceptible to enteroinvasion (p=0.02) and released less $sPLA_2$ in response to bacteria.

Conclusions: *EdnrB*-het and *EdnrB*-null animals initially demonstrate similar stool flora but then undergo reciprocal changes with time. *EdnrB*-null animals also demonstrate impaired mucosal defense, with decreased luminal sPLA2 and increased epithelial invasion of *E. coli* in vitro. These findings may explain susceptibility to recurrent HAEC and suggest a role for the neural crest in the development and regulation of gastrointestinal mucosal immunity.

INTRODUCTION

Hirschsprung's disease (HSCR) is characterized by congenital segmental aganglionosis of the enteric nervous system (ENS) in the distal gut due to a failure of neural crest cell (NCC) migration during embryonic development [1, 2]. The HSCR phenotype can be surgically treated by resecting the aganglionic bowel and performing a surgical "pull-through" of ganglionated bowel. However, up to 60% of treated patients continue to suffer life-threatening Hirschsprung's-associated enterocolitis (HAEC) throughout life [3-5], suggesting susceptibility is not dependent upon the presence of an obstruction alone. Clinical and animal investigations of HAEC have attempted to identify causative organisms that promote enterocolitis development [6-9]. These studies have not isolated a single, causative organism, but rather multiple investigators have identified a handful of potential contributors, including *Clostridium difficile*, *Escherichia coli*, and viruses, to which the host is susceptible in HAEC. An investigation of host mucosal barrier dysfunctions leading to pathogen susceptibility, such as the loss of antimicrobial production and secretion by Paneth cells, has not previously been undertaken in the context of HAEC.

The antibiotic metronidazole, an imidazole with predominant activity against anaerobic bacteria, is effective in HAEC management, which suggests that changes to anaerobic populations are central to disease etiology. The study of microbiome changes contributing to enterocolitis is made possible by the recent development of culture-independent analysis, since culture-dependent methods are limited to strains that can be grown under current laboratory protocols [10]. Culture-independent techniques examine all bacterial strains present in the gut by sequencing the bacterial 16S ribosomal RNA to analyze microbial diversity [11, 12]. These techniques, which employ bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), have

been used to investigate many pathological processes, including obesity, diabetes mellitus, inflammatory bowel disease, and necrotizing enterocolitis [13, 14]. These investigations demonstrate etiologies with consistent shift in microbiome composition - with relative over-representation or under-representation of specific genera and species rather than implicating a single causative organism. For instance, greater levels of *Lactobaillaceae* [15] appear protective due to anti-inflammatory components in their cell walls and their competitive exclusion against otherwise pro-inflammatory genera, such as *Bacteroidaeae* [16].

The microbiome population of the intestinal lumen is under tight regulation by the mucosa under normal circumstances [17]. Paneth cells, which reside at the base of the intestinal crypts, secrete a mileu of antimicrobial molecules including secretory phospholipase A_2 ($sPLA_2$), lysozyme, and defensins (cryptidins in rodents) [17, 18]. These compounds regulate the microflora and prevent colonization of the intestinal mucosa by pathogenic organisms. $sPLA_2$ functions to disrupt bacterial cell membranes in gram-negative and gram-positive bacteria [19-23]. Others have shown that the loss of Paneth cell antimicrobial products is associated with dysbiosis of the intestinal microbiome and increased tissue inflammation preceding colitis [24-27].

Our understanding of HSCR development in regard to colonic aganglionosis and gut development is advanced by the use of murine models. We previously reported our conditional knockout model Endothelin receptor B (*EdnrB*) null (*Ednrb* flex3/flex3/null) that contains deletion of EdnrB specific to enteric neural crest cells (ENCCs) [1, 28]. These *EdnrB*-null mice develop enterocolitis at approximately post-natal day 24-26 (P24 – P26) that is lethal at approximately P28. To investigate changes to the microbiome and functional changes to Paneth cells, we examined *EdnrB*-het and *EdnrB*-null animals at two time points: P16-P18 when both

sets of animals are developing normally and at P21-P24 just prior to the onset of colitis in the *EdnrB*-null animals. We observed the microbiota composition of *EdnrB*-null animals differs significantly from that of the *EdnrB*-het animals prior to enterocolitis, with a shift in the microbial composition of null animals characterized by increased *Bacteroidaceae* and *Closteridiaceae* and reduced *Lactobaillaceae* immediately preceding the onset of enterocolitis. We also demonstrate that EDNRB-null animals have an increased susceptibility to bacterial enteroinvasion, using an *ex vivo* intestinal segment culture (EVISC), that is consistent with concurrent loss of the Paneth cell antimicrobial compound, $sPLA_2$, in the small intestinal tissue and lumen.

MATERIALS AND METHODS

Animals

All procedures were approved by the University of Wisconsin Animal Care Committee. The mice were housed in a non-sterile environment and were allowed ad libitum access to food and water. We utilized a mouse model with neural-crest specific deletion of endothelin receptor B ($Ednrb^{flex3/flex3}$) [1, 28]. Mating the *TgWnt1-Cre/+ Ednrb$^{flex3/+}$* mice with *Rosa26$^{YFPStop/YFPStop}$ Ednrb$^{flex3/flex3}$* or *Rosa$^{26tdTomato\ Stop/tdTomato\ Stop}$ Ednrb$^{flex3/flex3}$* mice resulted in deletion of exon 3, the absence of a functional EdnrB and the presence of a fluorescent protein, either yellow fluorescent protein (YFP) or td Tomato, in neural crest cells. *EdnrB*-null (*Ednrb$^{flex3/flex3}$*) animals can be recognized by the absence of fluorescent ENCCs in their distal colon. The *Ednrb$^{flex3/+}$* mice are available from Jackson Laboratories (Stock Number: 009063). *EdnrB*-het littermates were used as controls. Mice were divided into 4 groups: *EdnrB*-null early (P16-P18), *EdnrB*-het

early (P16-P18), *EdnrB*-null late (P21-P24), and *EdnrB*-het late (P21-P24). The early time point is during normal development while the later time point is just prior to the onset of enterocolitis.

DNA extraction

Cecal stool samples were homogenized and 200mg aseptically suspended in 500µl RLT buffer (Qiagen, Valencia, CA) (with β- mercaptoethanol). A sterile 5mm steel bead (Qiagen, Valencia, CA) and 500µl volume of sterile 0.1mm glass beads (Scientific Industries, Inc., NY, USA) was added for complete bacterial lyses in Qiagen TissueLyser (Qiagen, Valencia, CA), run at 30Hz for 5min. Samples were centrifuged and 100µl of 100% ethanol was added to a 100µl aliquot of the sample supernatant. This mixture was added to a DNA spin column, and DNA recovery protocols were followed as instructed in the Qiagen DNA Stool Kit (Qiagen, Valencia, CA) starting at step 5 of the Protocol. DNA was eluted from the column with 50µl water and samples were diluted accordingly to a final nominal concentration of 20 ng/µl. DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France).

Massively parallel bTEFAP

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as described previously [10, 12, 29] using Gray28F 5'TTTGATCNTGGCTCAG and Gray519r 5' GTNTTACNGCGGCKGCTG. Initial generation of the sequencing library utilized a one-step PCR with a total of 30 cycles, a mixture of Hot Start and HotStar high fidelity taq polymerases, and amplicons originating and extending from the 28F for bacterial diversity. Tag-encoded FLX amplicon pyrosequencing analyses utilized Roche 454 FLX instrument with Titanium reagents,

titanium procedures performed at the Research and Testing Laboratory (Lubbock, TX) based upon RTL protocols (www.researchandtesting.com).

Bacterial Diversity Analysis

Sequencing data was analyzed by MR DNA (www.mrdnalab.com) after trimming low quality ends <Q25 and removing primers and tags, all failed sequence reads, sequences without 100% identity to expected barcode, sequences less than 200 bp in length, sequences with degenerate/ambiguous bases, and sequences with homopolymer stretches > 6bp were removed. Sequence collections were de-noised, de-multiplexed, and chimeras removed using USearch and UChime (Drive5.com). Sequence data was then clustered into operational Taxonomic units with 3% divergence using uClust. To determine the identity of bacteria in the sequence collection, sequences were de-noised, assembled into clusters and queried with BLAST+ against a database of high quality bacterial 16S rRNA sequences derived from GreenGenes (10-2011 version). Using a .NET and C# analysis pipeline the resulting BLASTn outputs were compiled, validated using taxonomic distance methods, and data reduction analysis performed.

Taxonomic Determination

Based upon the BLAST+ derived sequence identity, bacteria were classified at the appropriate taxonomic levels using the following criteria. Sequences with identity scores to known or well characterized 16S rRNA sequences, greater than 95 % were resolved to the level of genus level, 90-95 % to the family level, 85-90 % to the order level, 80-85 % to the class level and 77-80 % to the level of phyla. In this report, we use the analysis at the genera level, although our other data is available upon request. After resolving based upon these parameters, the

percentage of each bacterial ID was individually analyzed for each sample providing relative abundance information within and among the individual samples. Evaluations presented at each taxonomic level, including percentage compilations, represent all sequences resolved to their primary identification or their closest relative, as has been previously described [30]. Organisms identified with sequence reads representing less than 1% of the total population (ie. <10 sequence reads per 1000 total sequence reads) were excluded from the analysis. To assess the diversity of the GI microbiota, the Shannon-Weaver, chao1, and rarefaction (OTU) diversity indexes were calculated using the core pipeline of QIIme [31, 32] Alterations of microbial communities among the 4 groups was investigated using principal coordinate analysis (PCoA) based on the phylogeny-based Unifrac distance metric [33, 34]. PCoA graphs were plotted using 3-D plot (PAST). Anova with tukey-kramer post hoc was performed with xlstat (Addinsoft, NY). Dual dendrograms were constructed with NCSS 2007 (Kaysville UT).

Continuous fluorescent assay for sPLA$_2$ Activity

Fluorescent assay for sPLA$_2$ activity was performed as described previously (*Tsao et al*) [35], with modification to substrate preparation [20]. This assay uses a specific probe, Bis-BODipy FL, which is designed to fluoresce when the Sn$_2$ position of the phospholipid glyceraldehyde backbone is cleaved. After the reactions reached equilibrium temperature, the reaction curve was fit to a second-order polynomial equation and the first-degree coefficient was taken as the initial rate of reaction (expressed as change in Fl/min/uL sample). Blank wells containing only substrate and buffer were used to find coefficient rates determined as background activity. This method was established as a high throughput method to rapidly analyze sPLA$_2$ activity, and we have previously utilized this technique with small intestine secretions and tissue [20].

Bacterial Preparation for Ex Vivo Intestinal Segment Culture

Escherichia coli 5011-Lux containing ampicillin resistance were grown in 40 mL lysogeny broth (LB) for 48 hours at 37 ºC under 5% CO_2, then a surface sample was passed to a new 40 mL LB broth and grown for 24 hours at 37 ºC under 5% CO_2. The surface sample is used because bacteria growing near the culture surface express virulence. Bacteria were spun down at 1780 x *g* for 11 minutes, the supernate was poured off, and the pellet was re-suspended in 40 mL LB to wash twice, and then re-suspended in 1 mL DPBS at 4 ºC as a bacteria stock solution. A 1:100 dilution of the bacterial stock solution was made and read on a spectrophotometer (DU-640, Beckman, Urbana, IL) at 450 nm wavelength. Bacterial concentrations were adjusted based on growth curves previously established.

Ex Vivo Intestinal Segment Culture

Intestinal segments from the distal 10 cm of ileum, excluding Peyer's patches, were placed on a sterile surface covered in a light layer of RPMI and carefully opened longitudinally. Tissue glue (Dermabond, Ethicon, Cornelia, GA) was applied to the surface of a tissue disc and the intestinal segments were attached to the surface apical side up. Tissue discs were manufactured from polystyrene with a 9 mm outer diameter. Once the glue set (approximately 10-15 seconds), the tissue disc was lowered into a cell culture insert (Cat 3292, 3.0 µM pore, 12 well format, BD bioscience, NJ). Gentle pressure was applied to ensure adherence of the bottom tissue disc to the cell culture insert. Cell culture inserts were placed into 12 well plates prefilled with 1 mL RPMI+Ampicillin per well.

400 µL of bacterial inoculums ($1x10^8$ Colony Forming Units (CFU)/mL) in RPMI+Ampicillin was placed in appropriate wells for 1 hour at 37 ºC. Following the bacterial

challenge, the bacterial inoculum was collected, centrifuged at 14,000 x g for 2 minutes to pellet bacteria, and the supernate was stored at -80 ºC for analysis of mucosal secretions. The wells were rinsed 3 times with 400 µL of DPBS. Then 600 µL RPMI+Gentamicin was added to each well for 1 hour at 37 ºC. This was done to kill any remaining bacteria in the well or adherent to the mucosal surface. RPMI+Gentamicin were then removed and the wash step repeated. Then, 500 µL of 0.1 % Triton-X in PBS was added to each well. The 12 well plates were placed on an orbital shaker and agitated (175 rpm; New Brunswick Scientific Classic Series C1 Shaker) for 30 minutes at room temperature. Serial dilutions (10^1-10^7) of the cell lysate made in DPBS and plated on LB+Ampicillin agar plates that were grown for 18 hours at 37 ºC. Enteroinvasion was assessed by determining CFUs. RPMI+Ampicillin added to the 12 well plate was also plated to detect cell culture insert leaks. If leaks were detected from the apical to serosal side, the well was discarded from the experiment.

Quantification of sPLA2 in Ileum segments

To determine the amount of sPLA$_2$ in Paneth cell granules, we performed immunohistochemistry (IHC) on the ileum segments, as previously described [36]. Briefly, samples were fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, and stored at 4 Cº until processing. Samples were processed in a Tissue-Tek V.I.P tissue processor. Samples were then embedded in paraffin, sectioned at 5 µm with a microtome, and placed on Adhesive Coated Slides (White Aminosilane, Newcomer Supply, Madison, WI). Antigen retrieval was performed by boiling slides in 10 mM sodium citrate buffer (pH 6.0). Samples were incubated with primary antibody (group II sPLA2 (G-15) goat polyclonal IgG, sc-14468, Santa Cruz Biotechnology) overnight in 1% BSA-PBS at 4 C in a humidity chamber and in the dark with secondary antibody

(Alexa Fluor 594, donkey anti- goat IgG, 2 mg/mL, A11058, Invitrogen, Grand Island, NY) for 30 mins in 1% BSA-PBS at room temperature. DAPI (P36935, Invitrogen) was placed on each slide before covering to image nuclei. After imaging, densitometric measurements of sPLA2 intensity were quantified in 12 individual crypts per animal using NIH ImageJ software (version 1.43, http://rsbweb.nih.gov/ij/).

RESULTS

Microbiome Taxonomic Composition

To visualize microbiome changes at the genus level, a dual dendrogram of the top 43 predominant genera in the 4 experimental groups was generated for each animal and the relative similarity of their bacterial communities are shown under treatment [Figure 1]. Wards minimum variance clustering with weighted Manhattan distances was utilized to provide a dual hierarchal clustering of the samples and the predominant genera. Sample clustering is based upon the relative similarity of the bacterial community in each sample (samples with similar communities group together), while genera are clustered based upon the relative similarity of their distribution among samples. The relative percentage of respective genera in each sample is represented in the heat map, with the genera percentage color coded according to the legend in the upper left. The relative distances for clustering of the samples are provided in the upper right legend of the figure, while the distances for clustering of the genera are provided in the lower left.

According to the clustering, 15 of the 22 animals at the early time point, from *EdnrB*-het and *EdnrB*-null, contain similar clustering of microbiome composition (central cluster). At the late time point, 11 of the 14 *EdnrB*-null late animals diverge together compared to the earlier

time point samples (blue; left cluster). In contrast, 11 of the 12 *EdnrB*-het late animals diverge together compared with the other groups (red; right cluster), demonstrating a notable difference in the *EdnrB*-late animal's microbiome composition. Changes to individual genera across each experimental group are also displayed with variance and statistical differences [Table 1].

Changes to the top 3 predominant genera in all four experimental groups were assessed with ANOVA. The *EdnrB*-null animals at the late time point diverge only minimally from the early time point; however, more dramatic changes are evident when comparing the *EdnrB*-null and *EdnrB*-het animals at the late time point [Figure 2]. *Lactobaillaceae*, indicated as protective to the host mucosa through direct anti-inflammatory effects and through competitive exclusion of pathogens for mucosal access [15], are relatively similar between groups at the early time point [Figure 2a]. However, at the late time point, *Lactobaillaceae* represent roughly 80% of the microbiome in *EdnrB*-hets compared with only 15% in *EdnrB*-nulls. This suggests *EdnrB*-hets develop a protective microbiome, while the *EdnrB*-nulls fail to maintain protective *Lactobaillaceae* levels. In addition to the loss of *Lactobaillaceae* in *EdnrB*-nulls at the late time point, reciprocal changes are evident in the two other bacteria genera, *Bacteroidaceae* [Figure 2b] and *Clostreidiaceae* [Figure 2c], which are associated with mucosal inflammation[16]. At the early time point, the levels of these genera are between 8 and 15% in both animal groups. At the late time point, while the level of *Clostreidiaceae* is not completely repressed in *EdnrB*-hets, the percentages of *Bacteroidaceae* and *Clostreidiaceae* in *EdnrB*-nulls are high and significantly elevated compared with *EdnrB*-het samples, suggesting a relative over-representation of these two species immediately before the onset of colitis.

To further visualize differences in microbiome composition at the genera level across samples, principle coordinate analysis for each animal was generated using unifrac analysis

[Figure 3] based on primary and secondary vectors (graphed in the X, and Y, respectively). Samples are colored according to their experimental groups. The *EdnrB*-het late (green) is uniquely clustered on the far left X-axis (primary), compared to *EdnrB*-het early (Blue) and *EdnrB*-null early (yellow) and late (red). This analysis supports the dual dendrogram (Figure 1) results that demonstrate unique clustering for *EdnrB*-het late compared to the other experimental groups.

Microbiome Sequence Diversity (alpha diversity)

The alpha diversity of the microbiome was accessed using 3 different methods: rarefaction (Operational Taxonomic Unit (OTUs) at a level of sequence similarity >97%), Chao1, and Shannon indices [Figure 4]. These indices are used to estimate species number (OTU) and diversity, including richness (Shannon, Chao1) and evenness (Shannon), for each experimental group. These analyses demonstrated each of the four experimental groups contained between 900-1100 observed species and that there is no significant diversity differences between groups. In contrast to the possibility that enterocolitis is correlated with bacterial overgrowth, these findings suggest the total number of bacterial species and diversity of the microbiome are not changed, but rather, the composition of the microbiome community is altered.

Small Intestinal tissue and lumen sPLA$_2$ levels

To determine the expression of the Paneth cell antimicrobial compound sPLA$_2$ in ileum, immunohistochemistry was performed at the early and late time points in both groups [Figure 5b]. Tissue expression of sPLA$_2$ was obvious in both *EdnrB*-het and *EdnrB*-nulls at the early time point (P16). However, by the late time point (P22), sPLA$_2$ expression in *EdnrB*-null animals

was no longer visible, while *EdnrB*-hets continued to express high levels. Bright field analysis

also showed a reduction in the number of granules in Null late animals (data not shown),

suggesting degranulation of Paneth cells in *EdnrB*-nulls was responsible for the lack of tissue

sPLA$_2$ expression.

Since major changes in composition occurred between the *EdnrB*-het and *EdnrB*-null at

the later time point (P22), we compared the luminal level of the antimicrobial Paneth cell product

sPLA$_2$ in the small intestinal wash fluid samples [Figure 5a]. Compared with the *EdnrB*-het late

(4,887 ± 423 FL/min/uL), sPLA$_2$ activity was significantly decreased in the *EdnrB*-null late

animals (2,817 ± 600, p=0.009), suggesting effective impairment of host defense prior to the

onset of enterocolitis in *EdnrB*-nulls.

EVISC and Bacterial Enteroinvasion

To determine functional differences in susceptibility to bacterial enteroinvasion between

EdnrB-hets and *EdnrB*-nulls at the late time point (prior to colitis), ileum segments from both

groups were challenged with enteroinvasive *Escherichia coli*. We observed ileal segments form

EdnrB-nulls (42,400 ± 11,582 recovered CFUs/segment) were significantly more susceptible to

bacterial enteroinvasion than *EdnrB*-hets (10,133 ± 2,064, p < 0.04) [Figure 6a]. Additionally,

consistent with the loss of luminal sPLA2 activity, the ileum segments from *EdnrB*-nulls (561 ±

160 Fl/min/µL) released significantly less sPLA$_2$ in response *in vitro* to the presence of bacteria

in culture than *EdnrB*-hets (1,330 ± 192, p < 0.01) [Figure 6b]. These findings suggest a

functional defect in antimicrobial defense associated with increased mucosal susceptibility to

enteroinvasion in *EdnrB*-null animals just prior to the onset of colitis that is not observed in the

EdnrB-het animals.

DISCUSSION

Hirschsprung's disease (HSCR) is characterized by congenital segmental aganglionosis of the enteric nervous system (ENS) in the distal gut following a failure of neural crest cell (NCC) migration during embryonic development [1, 2]. The lack of ENS innervation of smooth muscle results in a loss of motility preceding a functional obstruction and severe intestinal inflammation known as Hirschsprung's-associated enterocolitis (HAEC). HSCR is routinely addressed surgically by resecting the aganglionic bowel segment and performing a surgical "pull-through" of the ganglionated bowel that removes the functional obstruction. However, up to 60% of patients continue to suffer from recurrent, severe life-threatening HAEC [3-5]. This signifies the etiology of HAEC is not dependent upon intestinal aganglionosis induced obstruction alone and that our understanding of this disease pathophysiology remains incomplete.

In this study we utilized a clinically relevant murine model of HSCR with our conditional knockout model for Endothelin receptor B (*EdnrB*) that contains deletion of *EdnrB* specific to enteric neural crest cells (ENCCs) [1, 28]. This model mimics the most common human aganglionosis and allows reproducible HAEC phenotypes with colitis onset at P24-26 that is lethal by P28. Using this model, we examined animals at an early time point (p16-18), 8 days before the onset of enterocolitis, and a late time point (p21-24), immediately prior to the onset of enterocolitis. We collected cecal stool samples for culture independent microbiome analysis, using 16S bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), which allows for a comprehensive characterization of the microbiome composition between groups and time points. To determine potential defects in mucosal defense, we collected small intestinal tissue and luminal rinse from *EdnrB*-het and *EdnrB*-null animals (P21-24) for measurement of the Paneth

cell antimicrobial protein sPLA$_2$. Furthermore, we performed a functional challenge of small bowel tissue from both groups using enteroinvasive *Echerichia coli*. These data demonstrate that *EdnrB*-null animals develop a pathogenic shift in the microbiome composition in contrast to a protective microbiome composition observed in the *EdnrB*-het mice. The *EdnrB*-null microbiome shift is concurrent with a loss of luminal and tissue sPLA$_2$ and an increased susceptibility to mucosal enteroinvasion.

Recent investigations have attempted to identify causative pathogenic organisms that might promote the onset or severity of HAEC [8, 9]. These studies demonstrate not a single, causative organism, but rather a shift in microbiome composition, including decreased levels of the genera *Lactobaillaceae*, which normally protects the mucosa through competitive exclusion of pathogens [15], and increased levels of the generas *Bacteroidaeae* and *Coprobacillus*, which are considered pro-inflammatory [7-9, 16, 37]. In agreement with the recent results of Ward *et al* [8] that utilized a global *EdnrB* knockout model, we observed a significant decrease in the total percentage of *Lactobaillaceae* in the *EdnrB*-nulls at the late time point. Concurrently, the level of *Lactobaillaceae* in EDNRB-het animals was significantly increased. Also consistent with Ward *et al* [8] we observed elevated levels of the percentage of the genera's *Bacteroidaeae* and *Clostreidiaceae* in the *EdnrB*-null animals at the late time point, while these genera were low in the *EdnrB*-het animals. Interestingly, between time points the change to the microbiome composition in *EdnrB*-nulls were not as dramatic as changes in the *EdnrB*-hets, but was characterized by a significant elevation in the percentage of pro-inflammatory bacteria and a significant decrease in the protective genera, *Lactobaillaceae*. In contrast, the *EdnrB*-het microbiome was characterized overall by protective compositional changes. Importantly,

microbiome shifts do not necessarily reflect disease etiology, rather a pathogenic microbiome shift with coexisting host susceptibility [38].

The intestinal microbiome is under constant surveillance and regulation by the host mucosa. Within the epithelium, specialized secreting cells produce antimicrobial compounds and mucins which protect the mucosal surface while allowing sufficient nutrient digestion and microbial presence [19, 39, 40]. Specifically, Paneth cells produce and secrete an array of antimicrobial peptides and proteins, including lysozyme, secretory Phospholipase A_2, RegIIIγ, and defensins or cryptidins[17-19]. Antimicrobial products from Paneth cells are major determinants of microflora populations and their loss is associated with mucosal inflammation and colitis [24-27]. Of these antimicrobial products, sPLA$_2$ functions catalytically to cleave fatty acids from glycerol backbones of phospholipids in cellular membranes. With an overall cationic charge, sPLA$_2$ is drawn to the negative charge on bacterial cell membranes within the gut lumen where it functions to induce membrane permeability and lysis [41]. sPLA$_2$ has been demonstrated to induce bacterial lysis of gram-positive and gram-negative strains *in vitro* and appears to preferentially attack membranes sites involved in growth[19, 21-23, 42]. The degranulation of Paneth cell products, including sPLA$_2$, into the lumen is regulated the binding of pathogen-associated molecular pattern (PAMP) to innate receptors, such as extracellular Toll-Like Receptors (TLRs) and intracellular Nucleotide- Oligermerization Domains (NODs).

In this study, we observed a significant reduction in Paneth cell sPLA$_2$ expression and level in the tissue and lumen of *EdnrB*-null animals just prior to the onset of enterocolitis, concurrent with the pro-inflammatory shift in microbiome composition. In contrast, the level of sPLA$_2$ was maintained in the *EdnrB*-hets. To our knowledge, the reduction in sPLA$_2$ in this HSCR model is the first report of Paneth cell dysfunction in HAEC. This finding suggests that

the *EdnrB*-nulls become susceptible to colitis through a loss of antimicrobial defense, either through by over stimulated PAMP receptors inducing Paneth cell degranulation or an intrinsic deficiency in phenotype. Mucosal defense defects have also been indicated for goblet cells, where decreased intestinal mucin levels were measured in the stool of human HSCR patients [43].

Considering the loss of Paneth cell antimicrobial expression in *EdnrB*-nulls, dramatic changes to the overall intestinal bacteria numbers might be expected. In contradiction to the hypothesis that enterocolitis results from bacterial overgrown or a reduction in community diversity as is seen in Crohn's colitis, our results demonstrated no change in total bacteria or species diversity and richness indices between groups. We instead observe under- and over-representation of certain species. Consistent with the presence of identified pathogens in previous HAEC investigations, including *Clostridium difficile* [44] and *Escherichia coli* [8], we observed elevated levels of *Echerichia coli* in EDNRB-null samples at the late time point that were not present in *EdnrB*-hets (Figure 1).

No previous work has investigated functional mucosal immune or epithelial changes in HSCR models, although such susceptibilities have been suggested [6, 45]. Therefore we examined mucosal differences between *EdnrB*-het and *EdnrB*-null in response to bacterial challenge; as has been performed in inflammatory bowel disease and necrotizing enterocolitis [46, 47]. Since we identified the presence of *Echerichia coli* in *EdnrB*-nulls, but not *EdnrB*-hets, we utilized an enteroinvasive strain of this pathogen for use in a functional challenge of intestinal mucosal segments from *EdnrB*-het and *EdnrB*-nulls. Strikingly, tissue segments from the *EdnrB*-null mice were 4 times more susceptibility to *in vitro* bacterial enteroinvasion by *Echerichia coli* than *EdnrB*-hets at the later time point. Ileal segments from *EdnrB*-nulls also released less than half the level of the Paneth cell antimicrobial protein $sPLA_2$ in response to bacterial challenge.

One limitation to this study is we cannot conclude if changes to *EdnrB*-nulls are due to the functional obstruction or intrinsic characteristics of the phenotype. For instance, alterations to Paneth cell function, as measured by the constitutively expressed antimicrobial sPLA$_2$, could be influenced by the obstruction, by the pathogenic shift in microbiome structure leading to over exocytosis of Paneth cell granules, or the phenotype. To explore these and other possibilities, we are currently developing a colostomy model to bypass the colonic obstruction and further test these possibilities and long-term gut mucosal immune structure and function. This model will allow resolution to these questions and limitations.

In summary, this study demonstrates pathogenic changes to the composition of the microbiome in *EdnrB*-null late animals immediately prior to lethal enterocolitis compared with *EdnrB*-het animals that develop protective microbiome structures. Further, the microbiome changes are characterized by shifts in composition, rather than a change in the absolute number of bacteria or species diversity. Interestingly, the pro-inflammatory microbiome changes in *EdnrB*-nulls were concurrent with the loss of the Paneth cell antimicrobial sPLA$_2$, which we speculate primes the mucosa to subsequent enterocoltis. This postulation is supported by the finding that the *EdnrB*-null *ex vivo* mucosa is more susceptible to opportunistic enteroinvasive pathogens. These data are the first demonstration of the potential role of defective host mucosal antimicrobial defense in susceptibility to HAEC. Further understanding of changes to the mucosal defense, such as effects upon Paneth cells, may allow targeted stimulation for prevention and treatment of HAEC.

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Figure 1.
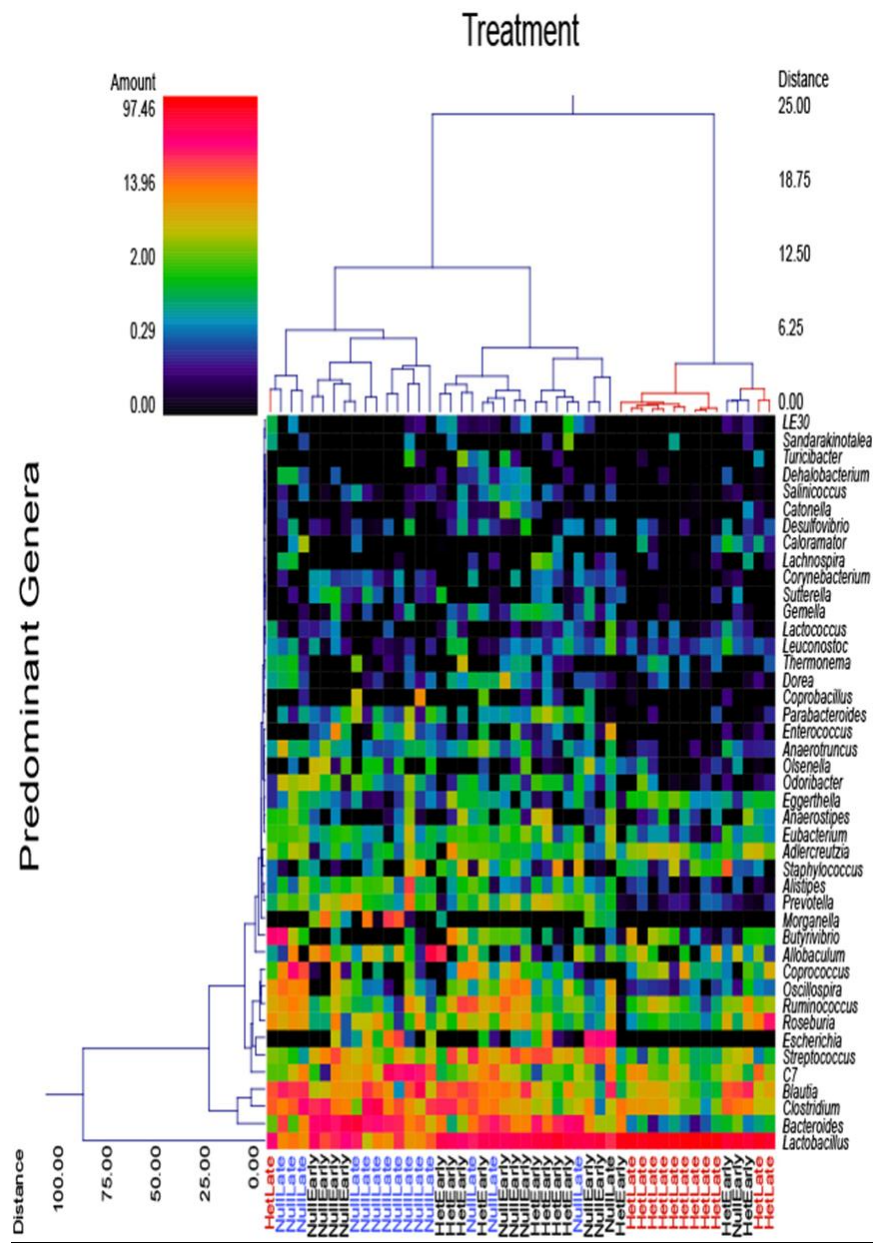
Dual Dendrogram evaluating the top 43 genera among groups. Wards minimum variance clustering with weighted Manhattan distances were utilized to provide a dual hierarchal clustering of the samples and the predominant genera. Sample clustering is based upon the relative similarity of the bacterial community in each sample (samples with similar communities group together), while genera are clustered based upon the relative similarity of their distribution among samples. The heat map indicates relative percentage of each genus within each sample.

Figure 2.
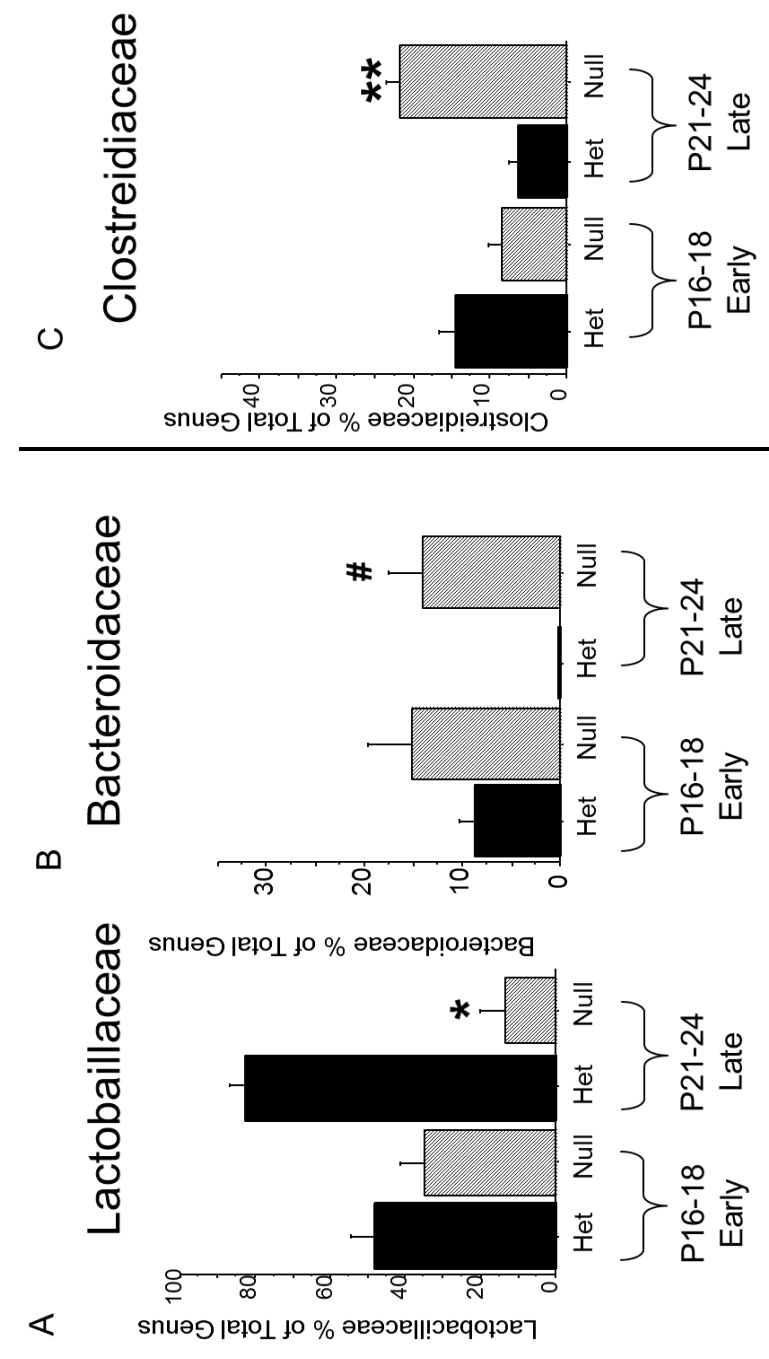
Relative abundance of the top 3 Bacterial population at the genera level. By bTEFAP analysis, *Ednrb*-het and *Ednrb*–null animals displayed similar flora at the earlier time point (P16-18). By later time points (P21-24), *Ednrb*-null animals demonstrated a decrease in *Lactobacillaceae* (a common probiotic) and an increase in *Bacteroidaceae* and *Clostreidiaceae* (common pathogens) over time. *Ednrb*-het animals demonstrated reciprocal changes. (*p<0.001, #p=0.001, **p=0.003)

Figure 3A.
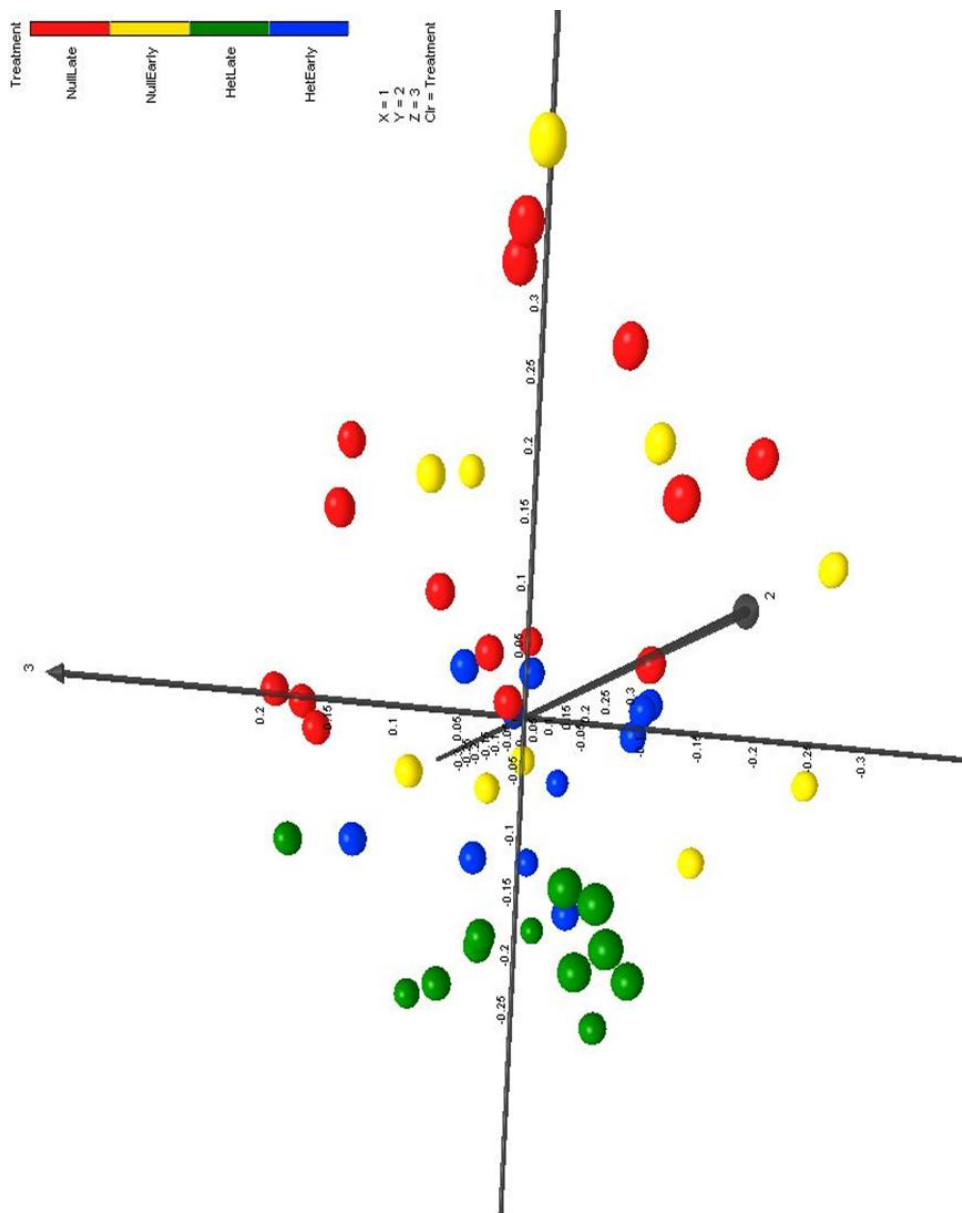
Principal coordinate analysis of Unifrac distances for sequences obtained from *EdnrB*-het and *EdnrB*-null animals at early and late time points. 3D representation of the first 3 principal coordinates. Axis percentages indicate the amount of variability explained by each coordinate. Ellipses indicate 95% confidence intervals for each group. Both figures show *EdnrB*-het at the late time point separate from the other 3 groups.

Figure 3B.
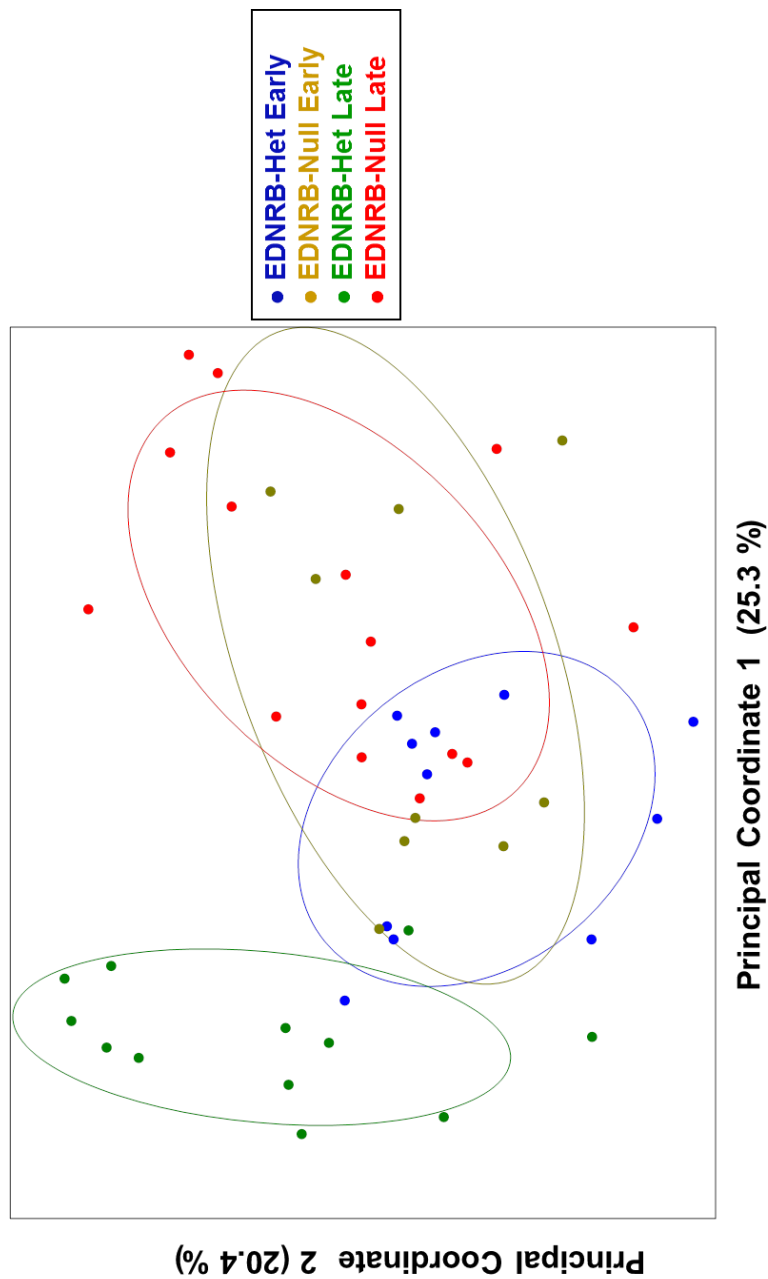
Principal coordinate analysis of Unifrac distances for sequences obtained from *EdnrB*-het and *EdnrB*-null animals at early and late time points. 2D representation of the first two principal coordinates. Axis percentages indicate the amount of variability explained by each coordinate. Ellipses indicate 95% confidence intervals for each group. Both figures show *EdnrB*-het at the late time point separate from the other 3 groups.

Figure 4.
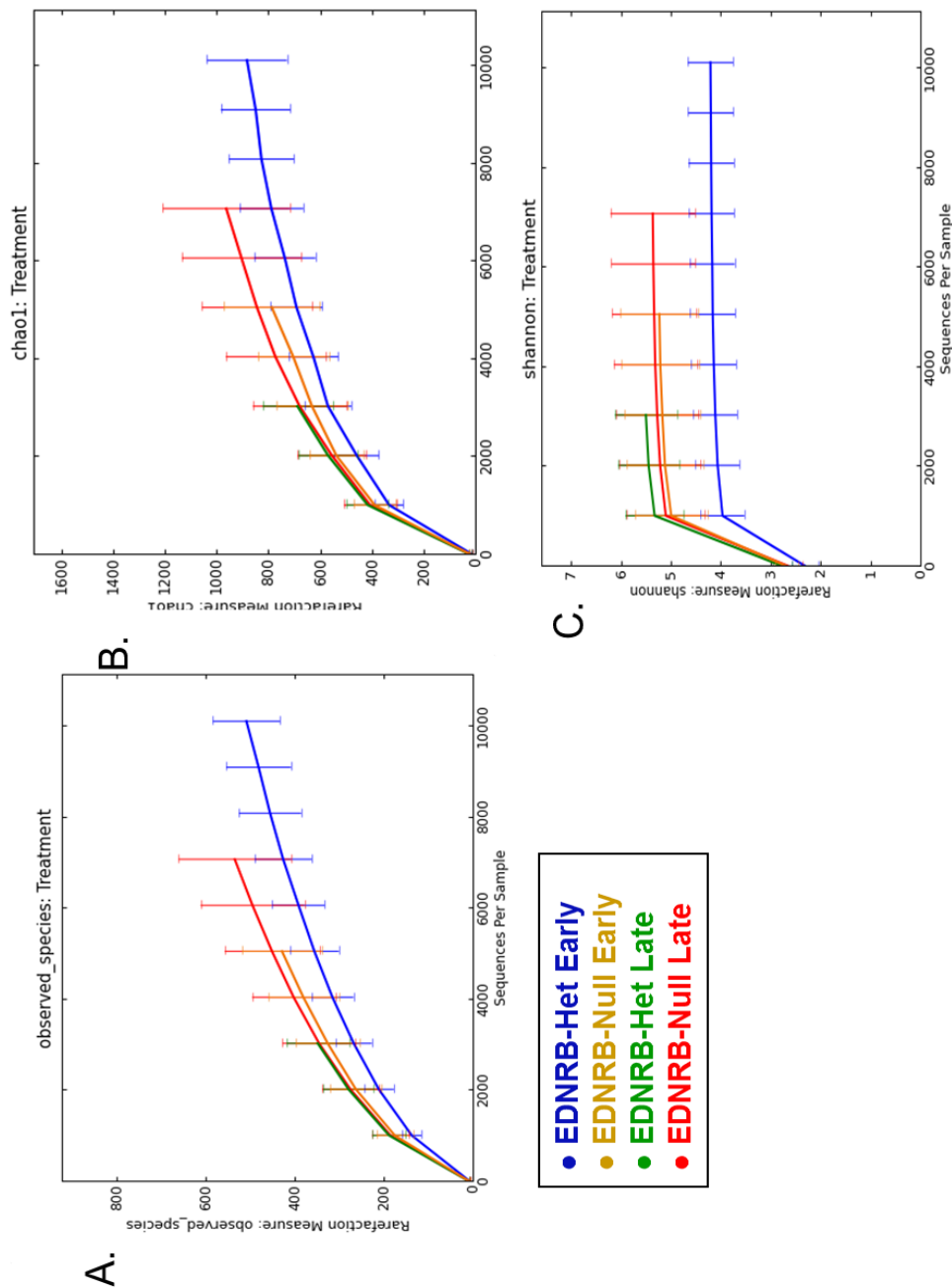
Number of operational taxonomic units (OTUs) and diversity (Shannon and Chao1) in cecal samples from *Ednrb*-het and *Ednrb*-null at the early and late time points. There were no significant differences between the groups in either the estimated number of species (A) or the diversity (B, C) of the microbiome communities between groups.

Figure 5A.
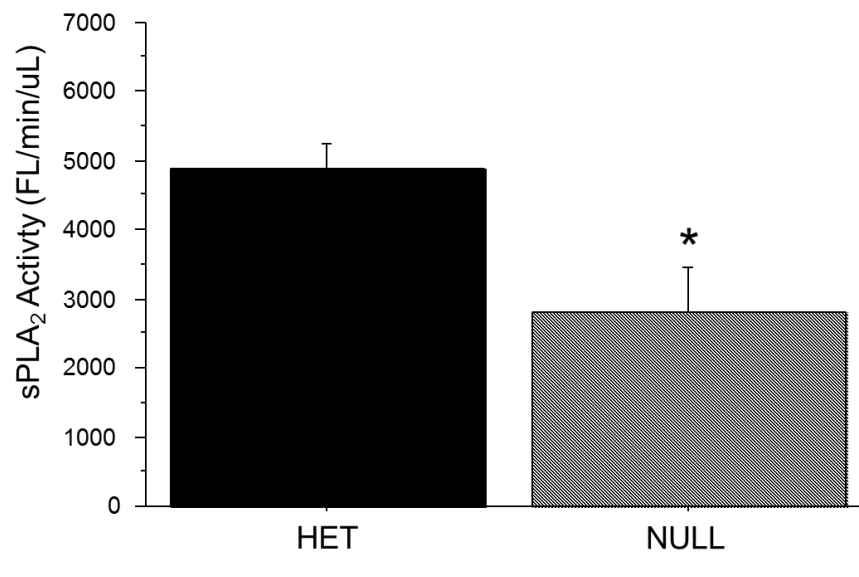
Small intestinal wash fluid sPLA$_2$ activity and tissue sPLA$_2$ expression. At P21-24, *Ednrb*-null mice had significantly decreased luminal levels of sPLA$_2$ activity compared to *Ednrb*-het. * p<0.01

Figure 5B.
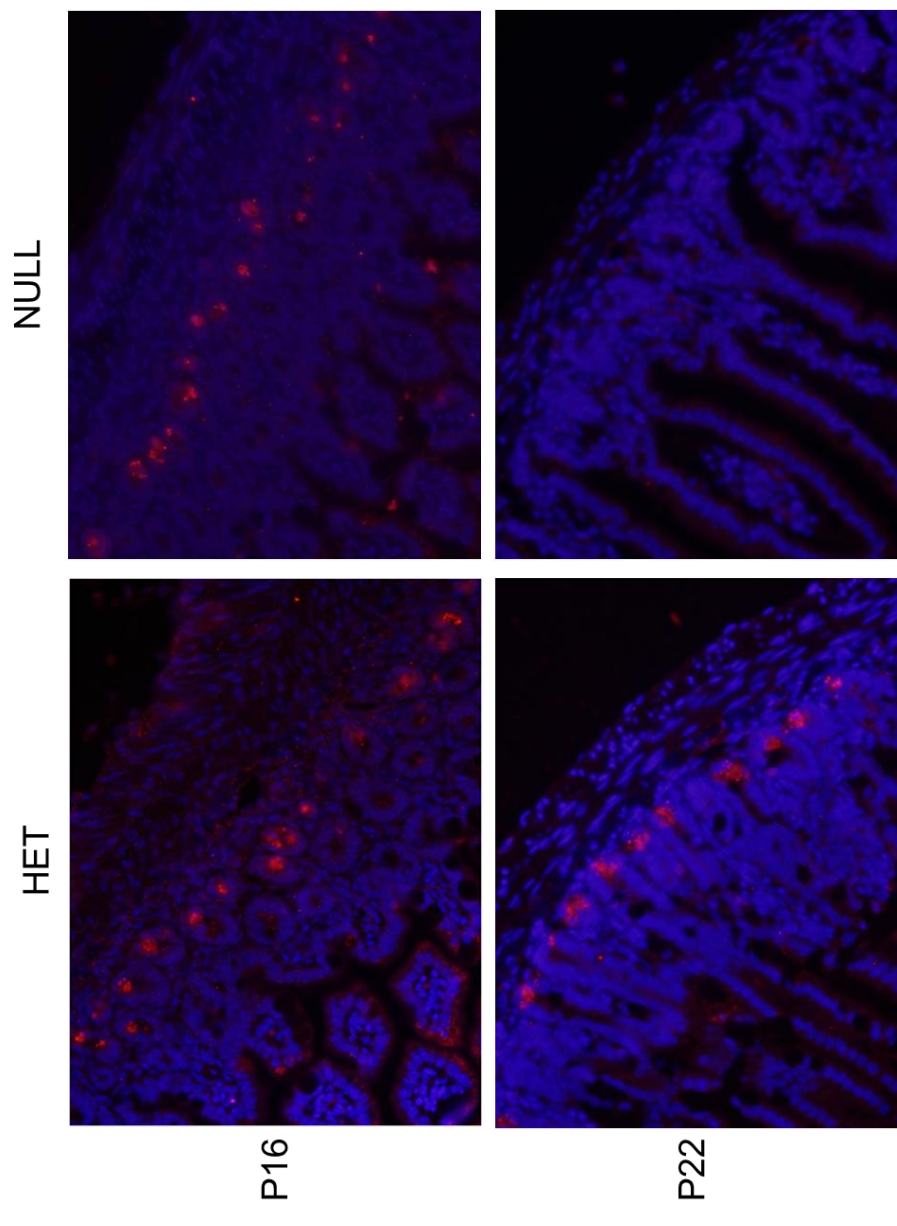
Small intestinal wash fluid sPLA$_2$ activity and tissue sPLA$_2$ expression. Representative immunohistochemical images of Paneth cell sPLA$_2$ (Red) with DAPI cell nucleus stain (blue) in ileum segments. sPLA$_2$ was expressed at the early time point (P16) in both animal groups, but was not visible in *Ednrb*-nulls at the late time point (P22).

Figure 6A.
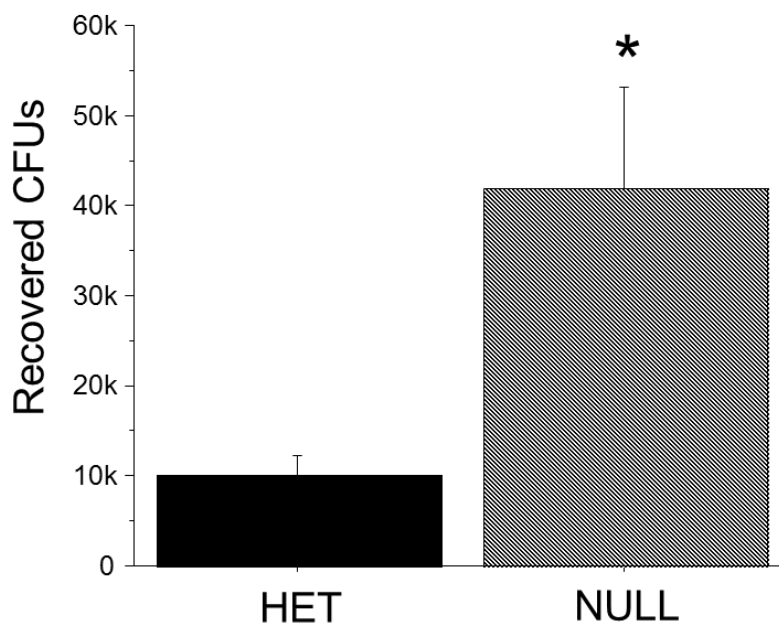
Recovered bacteria from tissue segments and sPLA$_2$ activity in secretions after *ex vivo* intestinal segment culture (EVISC). *Ednrb*-null segments had significantly more enteroinvasive *E. Coli* recovered compared to *Ednrb*-het, demonstrating increased enterinvasive susceptibility *p<0.05

Figure 6B.
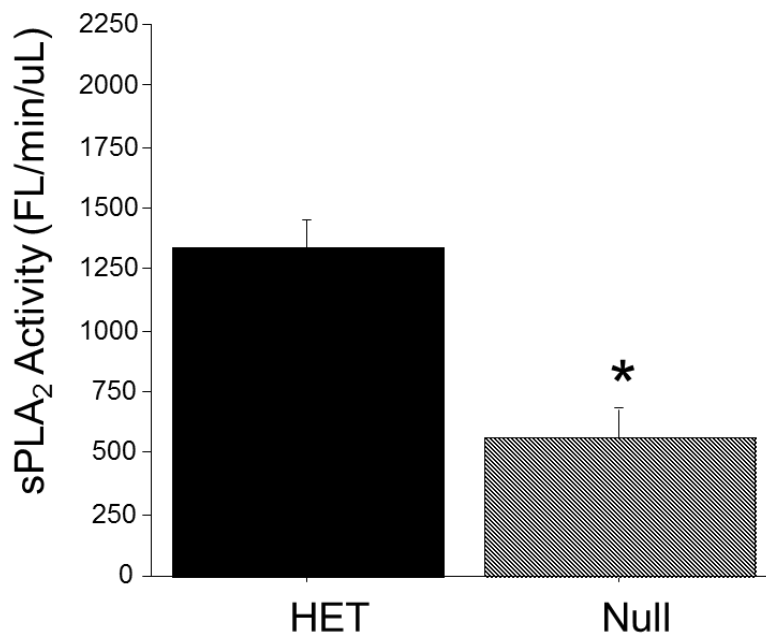
sPLA$_2$ activity from secretions of tissue segments during enteroinvasion challenge was significantly decreased in *Ednrb*-null segments compared to *Ednrb*-het (*p<0.05). Recovered bacteria from tissue segments and sPLA$_2$ activity in secretions after *ex vivo* intestinal segment culture (EVISC).

Table 1.

Genus	Ednrb-Het Early	Ednrb-Null Early	Ednrb-Het Late	Ednrb-Null Late
Lactobacillus	52.41 ± 4.35 ^{A}	39.11 ± 5.69 ^{A}	79.19 ± 5.36 ^{B}	14.08 ± 3.59 ^{C}
Bacteroides	9.71 ± 2.95 ^{AB}	16.89 ± 5.14 ^{A}	0.20 ± 0.06 ^{B}	15.91 ± 3.77 ^{A}
Blautia	7.43 ± 1.80 ^{AB}	5.33 ± 1.63 ^{AB}	2.45 ± 0.80 ^{B}	11.82 ± 2.75 ^{A}
Clostridium	7.27 ± 1.86 ^{AB}	7.49 ± 2.10 ^{AB}	2.76 ± 0.73 ^{B}	12.68 ± 3.54 ^{A}
Streptococcus	4.92 ± 1.45 ^{AB}	7.70 ± 1.43 ^{A}	0.60 ± 0.19 ^{B}	3.02 ± 1.15 ^{B}
Ruminococcus	2.60 ± 1.17 ^{NS}	2.09 ± 0.88	1.04 ± 0.27	2.76 ± 0.81
Staphylococcus	1.87 ± 0.94 ^{NS}	0.18 ± 0.84	0.36 ± 0.14	0.76 ± 0.40
Roseburia	1.67 ± 0.40 ^{NS}	2.06 ± 0.84	3.03 ± 1.82	2.09 ± 0.44
Allobaculum	1.55 ± 1.43 ^{NS}	1.81 ± 0.84	0.70 ± 0.28	4.77 ± 3.39
Eubacterium	1.14 ± 0.46 ^{A}	1.56 ± 0.51 ^{A}	2.33 ± 0.75 ^{A}	10.67 ± 2.92 ^{B}
Adlercreutzia	0.97 ± 0.46 ^{NS}	0.23 ± 0.06	1.19 ± 0.20	0.67± 0.13
Coprococcus	0.96 ± 0.56 ^{NS}	1.16 ± 0.60	0.94 ± 0.33	3.72 ± 2.06
Butyrivibrio	0.88 ± 0.55 ^{NS}	0.19 ± 0.10	3.33 ± 2.67	1.80 ± 1.54
Escherichia	0.86 ± 0.76 ^{NS}	5.04 ± 3.20	0.00 ± 0.00	3.74 ± 2.09
Prevotella	0.81 ± 0.27 ^{NS}	1.14 ± 0.35	0.05 ± 0.02	1.30 ± 0.57
Anaerostipes	0.66 ± 0.28 ^{NS}	0.10 ± 0.04	0.13 ± 0.05	0.30 ± 0.12
Oscillospira	0.61 ± 0.19 ^{NS}	2.44 ± 1.00	0.35 ± 0.30	1.84 ± 0.72
Eubacterium	0.47 ± 0.14 ^{NS}	0.40 ± 0.11	0.14 ± 0.05	0.55 ± 0.15
Parabacteroides	0.35 ± 0.15 ^{NS}	0.14 ± 0.05	0.01 ± 0.01	0.32 ± 0.12
Eggerthella	0.24 ± 0.12 ^{NS}	0.08 ± 0.02	0.41 ± 0.09	0.27 ± 0.09
Thermonema	0.20 ± 0.19 ^{NS}	0.05 ± 0.03	0.08 ± 0.03	0.12 ± 0.07
Anaerotruncus	0.20 ± 0.10 ^{NS}	0.23 ± 0.09	0.03 ± 0.01	0.36 ± 0.17
Alistipes	0.20 ± 0.07 ^{NS}	0.20 ± 0.06	0.03 ± 0.01	1.40 ± 0.88
Lachnospira	0.17 ± 0.10 ^{NS}	0.00 ± 0.00	0.01 ± 0.00	0.06 ± 0.03
Odoribacter	0.17 ± 0.06 ^{AB}	0.38 ± 0.19 ^{AB}	0.41 ± 0.09 ^{B}	0.60 ± 0.16 ^{A}
Leuconostoc	0.15 ± 0.05 ^{NS}	0.08 ± 0.01	0.06 ± 0.01	0.08 ± 0.05
Turicibacter	0.11 ± 0.09 ^{NS}	0.03 ± 0.03	0.01 ± 0.01	0.04 ± 0.04
Coprobacillus	0.09 ± 0.07 ^{NS}	0.03 ± 0.02	0.00 ± 0.00	0.46 ± 0.35
Morganella	0.02 ± 0.02 ^{NS}	1.13 ± 0.79	0.00 ± 0.00	2.18 ± 1.20
Olsenella	0.02 ± 0.01 ^{A}	0.72 ± 0.26 ^{B}	0.03 ± 0.02 ^{A}	0.16 ± 0.05 ^{A}

** Values are Mean ± SEM. Different superscript letters between values denote the groups are significantly different (P < 0.05). NS denotes no significant differences between groups. Differences were assessed using ANOVA with Tukey HSD post-hoc analysis.

APPENDIX II

Pharmaconutrition Review: Physiological Mechanisms

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Abstract: The search to improve outcomes in critically ill patients through nutrition support has steadily progressed over the last four decades. One current approach to this problem is the addition of specific nutrients to improve host defenses and improve the outcome of critically ill patients. The field is referred to as 'Pharmaconutrition' with the hope of focusing investigations on each nutrient to understand their pharmacological effects upon immune and clinical outcomes. The purpose of this review is to describe some of the known physiological mechanisms of pharmaconutrients such as glutamine, arginine, ω-3 fatty acids, and selenium.

"Let food be thy medicine and medicine be thy food" -Hippocrates

Introduction:

Critical illness poses significant challenges to patients who must respond effectively to systemic inflammation, potentially infectious organisms, altered immunity, and metabolic changes resulting in hypermetabolism. The inability to maintain adequate nutrient delivery during these hypercatabolic conditions renders the patient susceptible to significant nutrient deficiencies, which may increase the risk for infection, organ failure, and mortality [1-4]. Since the ability of patients to meet their needs by eating is usually not possible, the use of specialized nutrition support through parenteral and enteral feeding is frequently employed to provide the required amounts of caloric and nutrient needs. The field of specialized nutrition support has progressed in knowledge and complexity over the past 45 years from simply solving the problem of safely providing enough micro- and macronutrients to meet metabolic demands to the current search for optimal nutrients for immune function and cell recovery.

Initially, the search for specialized formulas focused on enteral products enriched with various combinations of glutamine, arginine, antioxidants, nucleotides, and/or ω-3 fatty acids. The enteral products were referred to as 'immunonutrition' or 'immune-enhancing diets' (IED) and were formulations or 'cocktails' of nutrients in various concentrations. Since these products contained different combinations of assorted nutrients, the exact contribution of each individual nutrient could not be determined in humans. In 2008, Jones and Heyland [5] suggested a shift from the concept of immunonutrition towards a standardized assessment of specific nutrients administered at pharmacological levels, coining the term 'pharmaconutrition'. This framework suggested nutrients be studied as pharmacological agents administered in physiological and

supraphysiologic doses, thus shifting the focus of nutrition support to a study of active therapeutics. The difficulty in defining optimal combination of agents and the optimal dose of each agent remains an impossible task. Nevertheless, it appears that specific pharmaconutrients do improve clinical outcomes in surgical and intensive care unit (ICU) patients.

There are conflicting results in IED data in regard to infection, morbidity, length of stay (LOS), mortality, and other outcome parameters. Potential explanations for discrepancies include variable effects of individual nutrients dependent on dose, the very real possibility of nutrient interactions, and that different hypermetabolic states may dictate different metabolic needs. Similarly, an individual's genome may influence metabolic requirements and immune response. Each factor limits our ability to evaluate individual nutrient effects. For example, arginine supports lymphocyte function but is also a nitric oxide precursor. Therefore, in addition to other confounding variables, arginine may improve outcomes in one type of patient while potentially harming another. While the controversy regarding arginine is heatedly debated and the conclusions are controversial, clinicians must be mindful that substances potent enough to help can also be potent enough to harm and consider each nutrient with full regard to dosage, route, timing, and duration in distinct patient subpopulations. The purpose of this review is to cover potential beneficial physiological mechanisms for several individual 'pharmaconutrients' and briefly describe the current clinical data from critically ill and surgical patients.

Macronutrients:

Glutamine

Glutamine is the most abundant free amino acid in circulation (500-900 μmol/L) under normal circumstances [6]. Under conditions of critical illness or with gastrointestinal disorders, glutamine levels may not be adequate to meet demands and becomes conditionally essential [7-9]. During critical illness, muscle cells metabolize branched chain amino acids obtained from intracellular protein catabolism [10-12]. As the branched chain amino acids are deaminated, nitrogen is transaminated onto either pyruvate creating alanine or onto alpha-ketoglutarate (which is produced in the Krebs cycle) that assimilates two ammonia groups to produce glutamine. Although alanine and glutamine constitute only 7% of normal muscle protein, they account for 60-70% of the amino acids released into the circulation by stressed or septic muscle cells. Glutamine is central to cellular energy, cell proliferation, renal acid-base regulation, and nitrogen and carbon metabolism [13, 14]. Glutamine also supports regulation of glucose metabolism through improved insulin sensitivity in trauma patients and provides carbon backbones required for glucose production through gluconeogenesis [15].

Within the intestine, glutamine is particularly important as the preferred fuel source by enterocytes and for the vast mass of immune cells within the gut-associated lymphoid tissue (GALT) [16-19], where up to 50% of available glutamine is metabolized following enteral feeding. Once activated, immune cells such as lymphocytes and macrophages utilize increased quantities of glutamine for energy, in addition to glucose [20]. Glutamine is also necessary for purine and pyrimidine synthesis of DNA and messenger RNA during immune cell proliferation [21]. Limited glutamine availability prevents expression of membrane receptors and cytokines [22], suppressing the efficiency of immune response. Since glutamine is utilized as a fuel source by enterocytes, colonocytes, and other cells in the gut mucosa, glutamine depletion impairs gut architecture and increases gut barrier permeability [16, 20, 23]. Maintenance of enterocyte absorption with enteral

glutamine may explain the clinical observation of increased formula volume tolerance in patients with glutamine supplementation [24]. Glutamine is also important in nervous system function, since it crosses the blood-brain barrier to serve as a chief precursor to the neurotransmitter gamma-aminobutyric acid (GABA) and glutamine supplementation enhances GABA concentrations [25].

In addition to metabolic contributions, glutamine is a precursor to the antioxidant glutathione, promotes heat shock protein (HSP) responses, and modulates gene regulation linked to apoptosis and signal transduction [26-29]. Glutathione expression is high in the intestinal mucosa and the reduced activity of this antioxidant leads to mucosal degradation, diarrhea, mal-absorption, and failure to thrive. Glutamine administration enhances glutathione levels [30-32]. The expression of HSP in particular is protective during cellular stress through prevention of cell damage and death in the splanchnic bed and other organs. The synthesis of key HSPs, including HSP-70, requires adequate glutamine levels and HSP-72 is demonstrated to correlate dose-dependently with glutamine availability in human mononuclear cells [33-36]. Laboratory experiments demonstrate glutamine administration decreases levels of circulating IL-6 and TNF-α and reduces NF-κB activity following sepsis [37], effects that are dependent upon HSP-70 [38]. Administration of glutamine in clinical and basic research settings reveal improved tissue HSP expression [28, 39]. Animal models of ischemia-reperfusion and sepsis demonstrate glutamine administration maintains metabolic function and survival of cardiac muscle and lung tissues [35, 40, 41].

Numerous clinical studies have examined the use of enteral and parenteral glutamine in critically ill patients. The most recent meta-analysis performed by the Canadian Critical Care

Nutrition Group (CCCNG) [42] examined twenty-one combined enteral and parenteral studies involving 1564 patients and determined a significant reduction in mortality from 20% to 14.7% (Risk Ratio (RR) with 95% Confidence Interval (CI): 0.75 [0.61, 0.93]). Improvements were also observed with LOS (RR with 95% CI: 0.79 [0.68, 0.93]) and infectious complication (RR with 95% CI: -2.56 [-4.39, -0.74]). Unfortunately, similar effects were not observed when only eight enteral studies involving 691 patients were examined alone, with the exception of LOS [42]. It should be noted that enterally fed patients generally represent a more heterogenic patient population compared with parenterally fed patients and this should be considered when critically interpreting clinical data. Two single-center clinical trials performed a decade ago in ICU patients examining parenteral glutamine observed significant reduction in mortality [43, 44], but this result was not confirmed in a more recent randomized prospective study of ICU patients receiving intravenous glutamine dipeptide (unpublished). Unfortunately, most clinical data utilized the more stable glutamine dipeptide that is not currently available in the United States. However, the use enteral Gln in severely burned patients decreased the rate of infection and improve gut function and survival [45, 46] and therefore current guidelines recommend enteral glutamine in burn and trauma patients [47, 48], but there is insufficient evidence to advise enteral glutamine in other critical illness. The route and dose of glutamine administration should be considered carefully, since enteral glutamine is rapidly metabolized by the splanchnic bed at low doses and may not reach the portal vein. The effectiveness of glutamine use with parenteral nutrition across all critically ill patient subgroups is still undetermined, but its use is recommended in all parenterally fed patients [47, 48].

Arginine

Arginine is a dibasic conditionally essential amino acid added to many pharmaconutrition formulations. In the urea cycle, arginine is derived from arginosuccinate and is further metabolized to produce urea and the amino acid ornithine. *De novo* biosynthesis of arginine utilizes citrulline as a precursor and citrulline is provided by glutamine metabolism in the intestine. Arginine is used in the biosynthesis of polyamines[49], proteins such as creatine and agmatine[50], and serves as the procurer for nitric oxide (NO; nitrogen monoxide), a potent vasodilator that is released in circulation with a half-life of several seconds [51-53]. In addition to precursor and biosynthetic roles, arginine stimulates certain physiological processes and is therefore also a regulatory molecule. For instance, elevated arginine levels increase collagen synthesis and growth hormone production in laboratory experiments, suggesting arginine may stimulate wound repair [49, 54]. Animal and cell culture models also demonstrate that high arginine concentrations improve glucose clearance and insulin sensitivity by influencing pancreatic beta-cells insulin release as well as glucose uptake by peripheral tissues in response to insulin [55, 56]. Arginine also regulates lymphocyte function, especially T cells [50]. *In vitro* cell culture conditions deficient in arginine result in arrested T lymphocytes cell development [57], while elevated levels of arginine stimulate lymphocyte proliferation through increased expression of surface T cell receptors (TCR) and cytokines [58, 59]. Arginine is required for lymphocyte memory following antigenic exposure and ζ-chain expression [60]. The ζ-chain, along with α/β or γ/δ heterodimers and CD3 make up the TCR complex required for coupled antigen recognition and low TCR complex expression results in impaired adaptive immune responses [60].

Arginine is the substrate for two myeloid cell enzymes that are up-regulated during immune activation: inducible nitric oxide synthase (iNOS) and arginase [50]. iNOS produces nitric oxide (NO), an important cell signaling molecule that regulates vasodilation of blood vessels and

vascular permeability. The importance of this role in vasodilitation is underscored by the finding that low levels of NO production can lead to ischemic organ damage and pulmonary hypertension [61, 62]. As an active antimicrobial compound within intra- and extra-cellular compartments, NO is also directly bactericidal and utilized by leukocytes and macrophages to destroy microbial pathogens [63]. During amplified immune response, iNOS expression and NO levels are stimulated in Th1 supporting macrophages (M-1) by pro-inflammatory cytokines, including IL-1, IL-2, TNF-α, and IFN-γ [64, 65]. Critical illness can lead to high levels of these pro-inflammatory cytokines that up-regulate iNOS expression of NO concentrations. Elevated NO levels are observed concomitantly in septic shock where hypotension, cardiac insufficiency, and increased tissue and endothelial permeability that may precede organ failure [66]. Cytotoxic effects attributed to NO include damage to cell structures, inactivation of metabolic pathways, lipid peroxidation, and alterations in gene expression [67, 68]. In contrast to iNOS, the other myeloid enzyme, arginase, converts arginine to ornithine and shunts available arginine away from NO production [50]. Arginase is expressed in Th2 supporting macrophages (M-2) following stimulation by Th2 anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and TGF-β [64, 69]. Ornithine produced by arginase can be used to synthesize polyamines and proline, which are needed for wound healing [70]. However, a key role for arginase during Th2 responses may be limiting NO production [71].

Recent findings suggest the type of response generated, Th1 or Th2, is influenced by the nature of injury [72]. For instance, trauma and major surgery patients appear to generate Th2 responses, whereas septic patients appear to generate Th1 responses [72]. Consistent with the role of arginine in NO production, some laboratory studies demonstrate negative effects with arginine supplementation in animal models of inflammation, infection, and injury [73, 74]. However, clinical

data suggest septic patients[75], but not trauma or elective surgery patients[76], may have elevated circulating NO levels, supporting the case for specific Th1 versus Th2 responses in specific injury. At least six meta-analysis studies have been performed on the available accumulative clinical data for arginine [77-81]. Each demonstrates reductions in overall infectious complications, four demonstrate reduced LOS, and two demonstrate decrease in ventilator days. In general, the data suggest most ICU patient and elective surgery populations may benefit from Arg with fewer infections. However, based upon data in sepsis, one highly controversial current recommendation is for withholding arginine supplementation in septic patients on the basis of potential harm induced by NO production and the increased formulation costs. Furthermore, hemodynamically unstable ICU patients should not receive additional arginine or enteral formulas in general [82].

Omega-3 (ω-3) Fatty Acids

Omega-3 (ω-3) fatty acids are isolated from cold water fish species, flaxseed, and canola oil and are used for their proposed anti-inflammatory properties [83]. ω-3 fatty acids are polyunsaturated fatty acids with a carbon-carbon double bond at the third carbon. Mammals cannot synthesize the carbon-carbon double bonds at either the third or sixth carbon, making these compounds essential in the diet [83]. The three most important ω-3 fatty acids are alpha-linolenic acid (ALA, 18 carbons, 3 double bonds), eicosapentaenoic acid (EPA, 20 carbons, 5 double bonds), and docosahexaenoic acid (DHA, 22 carbons, 6 double bonds). These ω-3 fatty acids are vital to normal cognitive function, but are also inserted in cell membranes where they compete with omega-6 (ω-6) fatty acids in the form of arachidonic acid (AA, 20 carbons, 4 double bonds) [5, 83-85]. Under healthy conditions, the ratio of ω-3/ω-6 fatty acids does not appear to have dramatic effects upon health. However, during cellular stress or damage these fatty acids

are released from the membranes by phospholipases and are converted by cyclooxygenase and lipoxygenase enzymes into powerful secondary messenger hormones, eicosanoids [86, 87]. Cyclooxygenase enzymes are the target of numerous non-steroidal anti-inflammatory drugs, such as aspirin, highlighting the significance of this common inflammatory pathway.

Eicosanoids derived from ω-6 fatty acids promote 2- and 4-series eicosanoids, such as prostaglandin E_2, prostaglandin A_2, and leukotriene B_4, which are highly pro-inflammatory. For example, these compounds mediate platelet coagulation, pain, redness, and swelling [86, 87]. Circulating prostaglandin E_2 functions directly upon the hypothalamus to induce fever, loss of appetite, and fatigue [88]. However, ω-3 fatty acids promote the less inflammatory eicosanoids of the 3- and 5-series, such as prostaglandin E_3 and leukotriene B_5. High doses of ω-3, or limited intake of ω-6, fatty acids appear to shift the balance in favor of less systemic inflammation in at least three ways [86, 87]. First, the ω-3 fatty acids displace AA so there is less ω-6 fatty acid proportionally within the cell membrane. Secondly, ω-3 fatty acids compete for cyclooxygenase and lipoxygenase enzymes, so less AA is catalyzed into pro-inflammatory eicosanoids. And third, the effect of 3- and 5-series eicosanoids directly counteracts the effects of the 2- and 4-series eicosanoids. Furthermore, ω-3 fatty acids decrease the release of pro-inflammatory cytokines IL-1 and TNF in peripheral blood mononuclear cells and reduces iNOS expression in macrophages [29, 89]. Considering the ω-3 fatty acid potential to reduce NO overproduction by limiting iNOS, adding ω-3 fatty acids to enteral formulas may benefit septic patients who are expressing Th1/M-1 inflammatory responses.

A clinical study revealed a rapid (<1 hour) and sustained (2-3 days) enrichment of leukocytes phospholipid ω-3 fatty acid content following intravenous bolus infusion in healthy

adults, demonstrating the effectiveness of ω-3 administration [90]. Due to the competitive effects between ω-6 and ω-3 fatty acids, the recommended ratio is 2:1-4:1 (ω-6:ω-3) [91] and this should be considered when interpreting clinical data. One unpublished and four published clinical trials examining ω-3 fatty acid formulations in critically ill patients have demonstrated beneficial outcomes [92-94]. Unfortunately, the formulations in these studies also include other compounds, such as antioxidant combinations, so there has not been a thorough analysis of ω-3 formulation effects alone. Even so, meta-analysis of these studies including 554 patients revealed significant benefits in mortality, where ω-3 treatment reduced mortality from 35% to 23.5% (RR with 95% CI: 0.67 [0.51, 0.87]) compared with the standard diets [42]. Further analysis of 340 critically ill patients from the same meta-analysis demonstrated significant reductions in LOS (RR with 95% CI: -4.49 [-6.49, -2.47]) and ventilator days (RR with 95% CI: -4.83 [-7.96, -1.70]) compared with standard diet. A recent clinical study with 106 septic patients that combined enteral ω-3 fatty acids with antioxidants demonstrated significant reductions in septic severity and cardiovascular and respiratory failure compared with controls [95]; however, there were no differences in overall mortality between groups. The use of ω-3 fatty acid in acute respiratory distress syndrome (ARDS) patients does appear to decrease mortality rates [96]. Additionally, improved pulmonary function and reduced lung edema have occurred with ω-3 supplementation in both clinical and laboratory studies [97, 98]. These findings led to the recommendation by ASPEN and CCCG to include enteral ω-3 fatty acids (with antioxidants) in patients with acute lung injury (ALI) and ARDS [42, 47, 48]. However, formulation, dosage, and duration remain controversial.

Leucine

Leucine is one of the three branched chain α-amino acids (BCAAs), along with isoleucine and valine. BCAAs are metabolized directly by skeletal muscle and, unlike other amino acids, do not reenter the circulation following muscle proteolysis. Leucine is the only dietary amino acid that promotes cell growth in the body [99]. Laboratory experiments demonstrate that leucine stimulates protein synthesis in skeletal, cardiac, and intestinal muscle, and other tissue and cell types through stimulation of the mammalian target rapamycin (mTOR) pathway [99, 100]. Animal experiments demonstrate that enteral leucine stimulates protein synthesis in a rat model of sepsis or burn [101]. Since critical illness is characterized by muscle catabolism and a 30% loss of lean muscle is associated with a significant increase in mortality [102-104], the addition of leucine to nutrition support formulas has the potential to slow muscle proteolysis in the critically ill.

BCAAs and their ketoacid derivatives have been used in clinical studies in the critically ill over the last 30 years. In burn patients, BCAA supplementation sustained mild inhibition of protein catabolism in some studies, as assessed by reduced urinary 3MH:creatine ratios, but these studies were confounded by small sample size and the use of all three BCAAs [105, 106]. Likewise, larger studies in trauma, ICU, and septic patients have been performed, where some showed improvements in nitrogen balance or the half-life of visceral proteins, but few improvements in mortality [107-110]. Furthermore, the vast majority of these studies utilized valine and isoleucine, in addition to leucine, and we now know that these other BCAAs do not stimulate protein synthesis and instead compete with leucine for transport into muscle cells and utilize shared metabolic pathways [111]. Considering these findings, more studies are needed that focus on leucine alone. De Bandt and Cynober [112] suggested future work should consider glutamine status when investigating leucine, since muscle proteolysis induced by low glutamine levels may override

any positive effects leucine has on protein synthesis and nitrogen balance. Larger controlled studies are needed to determine the effects of leucine, with balanced nutrition support, to determine its effect in respectively stratified critically ill patient subgroups.

Micronutrients:

Selenium

Selenium is an essential trace mineral required in μg quantities that is incorporated into the amino acid selenocysteine. It is an important co-factor in at least 25 selenoproteins, including important immune, endocrine, and antioxidants enzymes [113-115]. Selenium is most readily bioavailable in the form of inorganic salts, such as selenate and selenite [116, 117] and recent advances have been made in identifying novel biomarkers of selenium status [118]. The precise role of Se in humoral immunity remains unclear, but selenium deficient rodents demonstrate reduced lymphocyte function and decreased immunoglobulin (Ig)-M, IgG, and IgA production. Low selenium status in humans is associated with decreased IgG and IgM titers in circulation [119]. Likewise, macrophages and neutrophils rely upon selenoprotein dependent generation of reactive oxygen species (ROS) and reduced selenium availability decreases total neutrophil numbers [120, 121].

The most recognized role of selenium is the antioxidant function of glutathione. There are eight glutathione peroxidase (GP) isoforms, including cytosolic GPx1, gastrointestinal GPx2, and plasma GPx3 [114]. The plasma GPx3 correlates with the circulating form of selenium, Selenoprotein P (SePP), and these proteins are markers of selenium status [122, 123]. Deficiency of GPx3 and SePP are associated with increased mortality in septic clinical patients [123]. Clinical

data also demonstrates low GPx3 levels inversely correlate with C-reactive protein (CRP), procalcitonin (PCT), and the Sequential Organ Failure Assessment (SOFA) score [124, 125]. CRP is released by the liver as an acute phase protein and is a marker of inflammation that binds dying cells and activates complement. PCT is a precursor for calcitonin produced by the thyroid and neuroendocrine cells in the intestine and lung that respond to infection and systemic inflammation. Elevated levels of CRP and PCT suggest increased systemic inflammation when GPx3 levels are low. Selenium is also a cofactor in thioredoxin reductases (TRxR), enzymes found ubiquitously in mammals that are necessary for defense against oxidative damage and controlling the redox molecules hydrogen peroxide and NO [126]. Further, selenium is required for regulation of thyroid hormones through iodothyronine deiodinase proteins. Poor selenium status following trauma correlates with low thyroid T3 levels, effects that are reversed with selenium supplementation [127].

Clinical studies utilize mega dose selenium, typically around 1000 µg/day, with the aims of reducing infection and organ dysfunction. Such doses are high considering the World Health Organization (WHO) recommends an RDI at 70 µg/day and risk of toxicity at 900 µg/day. However, clinical data suggests selenium levels can decrease 40-70% during septic shock and serum levels <0.7 µmol/L are associated with significantly greater mortality and organ failure. Metabolic activity and urinary losses of nutrients are increased in critical illness and can be further accelerated by the use of statins and corticosteroids that suppress Se status [128, 129]. Considering some patients have preexisting dietary deficiency in addition to catabolic stress, the typical regimen is a bolus infusion followed by continuous infusion for 7-10 days, but not longer than 2 weeks [130]. Using this model, a clinical trial involving 189 septic and SIRS patients demonstrated significant reductions in mortality in several patient subgroups and treatment

significantly maintained whole blood Se markers [131]. Yet, there remains controversy over the safety of high dose selenium in PN and current CCCG guidelines [42] suggest there is insufficient data to make specific recommendations for the use of selenium in parenteral nutrition in critically ill patients, particularly considering its requirement and contraindications in specific patient populations, such as smokers and alcoholics where liver function may be impaired, is undefined. However, ASPEN [47] supports the use of selenium, and other antioxidants, in PN at lower doses for all critically ill patients requiring nutrition support. The current dosing range suggestion for the critically ill are 70-100 µg/L in EN and 100-400 µg/d in PN [132, 133].

Vitamin C (Ascorbic Acid)

Vitamin C is an important redox compound that reduces radical forms of α-tocopherol (Vitamin E), glutathione, hydroxyl, and superoxide as well as radicals in cell cytoplasm and mitochondria. Animal models demonstrate Vitamin C infusion protects again ischemia-reperfusion injury of the liver and skeletal muscle [134-136]. Other models have also demonstrated positive effects of Vitamin C administration in ARDS and SIRS that were attributed to free radical scavenging activity [137, 138]. In addition to limiting radicals, Vitamin C is important for the synthesis of collagen, carnitine, and norepinephrine and low levels of Vitamin C increase bruising and delay wound healing [139]. *In vitro*, Vitamin C reduces activation of NF-κB through TNF-α modulation [140] and spontaneously destroys histamine, a molecule associated with respiratory tract infection, allergic pathologies, and bronchial asthma [141]. Animal models demonstrate Vitamin C protects against vascular leakage, epithelial barrier disruption, and increased alveolar fluid levels during sepsis [138, 142]. Burn patients are subjected to increased oxidative stress and the need for increased collagen synthesis. A clinical study using high dose

Vitamin C in severely burned patients demonstrated reduced wound edema, resuscitation fluid volume requirements, and respiratory failure [139, 143, 144]. However, no large well-controlled studies have examined Vitamin C alone, without other antioxidants, upon immune function, vascular repair, or wound healing in other critically ill patients. Parenteral dosing of 200 mg/day is recommended during critical illness [48, 132]. The current enteral dose recommendation for Vitamin C is 125-250 mg/day, with supplemental recommendations of additional 500-3000 mg/d [47, 132]. Trauma and burn patients routinely receive elevated doses of 1000 mg/day [132] and some clinical data suggests 1000-2000 grams/day may be required to maintain normal levels of plasma Vitamin C (55-100 µmol/L) in postoperative ICU patients. Parenteral dosages of 3-6 grams/day for 2 days or 3 grams/day for several weeks may be beneficial without producing side effects or impaired renal function [143, 144].

Zinc

Zinc is a trace mineral with cofactor roles in catalytic, structural, and regulatory proteins throughout the body that are especially important in immune function and implicated in wound healing [145, 146]. Zinc deficiency can occur with high-calorie parenteral and enteral nutrition without appropriate zinc supplementation and may present with skin rash, abnormal sense of taste or smell, delayed wound healing, and the loss of hair [147-150]. In children, zinc deficiency can lead to failure to thrive. Zinc is a vital cofactor to DNA and RNA polymerase and zinc deficiency can cause result in arrested cell growth cycles [145, 151]. Clinically, deficiency can manifest as reduced natural killer cell numbers and function and greater risk of infection and mortality [147]. Human studies suggest temporary and mild zinc deficiency decreases the CD4:CD8 lymphocyte ratio and function [152]. In ICU patients, zinc plasma levels are inversely correlated

with the serum cytokines IL-6 and IL-8, which are indicative of inflammatory stress [153] . Zinc

deficiency *in vitro* increases the level of IL-1β and TNF-α in pro-myeloid cells through increased

redox stress [154]. Within the intestine, zinc has important antimicrobial roles as cofactors in matrix

metalloproteinase (MMP) enzymes, including MMP-7, which activates antimicrobial defensins

in intestinal Paneth cells [155]. Zinc also plays a structural role in intestinal tight-junction proteins,

which holds enterocytes and other epithelial cells of the mucosa together, and deficiency

increases mucosal barrier permeability and bacterial translocation [156, 157].

Zinc status is assessed by measuring serum concentration, which is normally lower than

levels in intracellular compartments; however, drug regimens can affect serum levels leading to

artificial status results. Other biomarkers for zinc status have been reviewed [158], but it remains

unclear which are appropriate in the critically ill. Leukocyte zinc levels may be a reliable

indicator of zinc status in the critically ill, but this analysis is challenging to perform [159]. The

normal range for serum zinc is 80-160 µg/dl and levels <60 µg/dl require zinc replacement.

Optimal dosing for zinc has not been determined [160], but recommended zinc intakes are 2.5-5

mg/day in parenteral nutrition, 11-19 mg/L in enteral nutrition, and an additional 12-30 mg/day

with diarrhea or fistulas [48, 132]. Up to 36 mg/day have been administered to burn patients without

deleterious side effects and careful monitoring of zinc status in fistula patients may be required

to ensure adequate status [161].

Magnesium

Magnesium is a divalent metal ion that is an essential cofactor for over 300 enzymes,

including those involved in protein synthesis, nucleic acid synthesis, and mitochondrial

membrane stabilization [162]. Magnesium deficiency is associated with dizziness, muscle cramping

and weakness, and fatigue. Renal reabsorption is usually highly efficient, but patients with poor renal function may be at increased risk of deficiency without magnesium supplementation [163]. Low magnesium levels are associated with significantly greater mortality in ICU patients and very low serum magnesium is associated with an increased incidence of cardiac dysrhythmias [164]. Accordingly, adequate magnesium is essential in preventing Idiopathic mitral valve prolapse (IMVP) and hypertension [165]. Additionally, similar to zinc, magnesium has essential roles in maintaining intestinal tight junction protein integrity and magnesium deficiency elevates liver and intestinal pro-inflammatory cytokine TNF-α and IL-6 levels [166]. Magnesium is also the cofactor for the enzyme arginase in myeloid cells and liver tissue [162]. There are currently no specific recommendations for clinical use, but magnesium status should be checked to assure proper administration in long term nutrition support patients.

Prebiotics, Probiotics, and Synbiotics

Probiotics are viable bacteria or yeast microorganisms of human origin that are proposed to benefit the host when added as dietary supplements [167]. *Prebiotics* are fermentable soluble dietary fibers, such as inulin and fructooligosacharides, which are metabolized by the microbiome and favor growth of "beneficial" bacteria in the gut [168]. *Synbiotics* is the term applied to nutrition products that contain both prebiotics and probiotics, with the aim of inducing additive beneficial effects in the host when consumed [168].

Although the specific mechanisms by which prebiotics and probiotics exert their effects remain loosely defined, it is likely they function largely by improving gut health and may provide stability in some conditions of critical illness during the use of antibiotics and decreased enteral intake [169, 170]. For example, at the most basic level specific probiotic organisms and

prebiotic substrates induce favorable shifts in intestinal microbiome composition, which outcompete pathogens for growth and mucosal attachment [171, 172]. Similarly, certain prebiotics and probiotics may improve the gastrointestinal barrier function directly by promoting goblet cell mucus production and secretion and release of selective anti-microbial products that protect the mucosa from adherent pathogenic microorganisms [173-178]. Since pathogens can influence absorption and ion balance at the mucosal interface, alterations in microbiome composition can result in diarrhea or bloating [179]. Probiotics also improve digestive function through increased nutrient availability and absorption, the production of short chain fatty acids that fuel colonocytes, and the synthesis of vitamins K, B12, thiamin, and riboflavin [180-182].

Recent interest has also focused on the immunomodulatary properties of pre- and probiotics, potentially through the GALT which contains up to 70% of immune cells in the body [183-187]. The use of prolonged parenteral nutrition reduces the number of Peyer's patch and lamina propria lymphocytes, mucosal Th2 cytokines, the mucosal sIgA transport protein, polymeric immunoglobulin receptor (pIgR)[188-191]. These effects result in reduced luminal sIgA and loss of the major limb of specific immunity in GI luminal fluid. sIgA is anti-inflammatory through bacterial opsinization, which prevents bacterial mucosal attachment. The presence of sIgA inhibits expression of virulence factors by gut bacteria [192]. Certain prebiotic and probiotic mixtures augment tissue anti-inflammatory cytokines and improve macrophage activation and antigen presentation, normalizing parameters of GALT function and stimulating the release of secretory Immunoglobulin-A (sIgA) on the gut lumen[193-195].

The study of pre- and probiotics and their effects upon human health is complicated by the extremely complex interactions that take place at the host-microbial interface. The human

intestine contains up to 100 trillion bacterial cells with the potential to influence expression of over 600,000 genes in addition to their effects upon GALT function [196]. Furthermore, microbial diversity and composition among individuals is highly variable and depends upon environment, diet, genetics, the source of colonization at birth, and the patients' global health. Despite these variables, 3 bacterial phyla dominate the microbiome composition: *Bacteroides, Firmicutes*, and *Actinobacteria* [197, 198]. Common probiotics include species from the *Firmicutes* and *Actinobacteria* phyla [196].

Clinical trials investigating the effects of pre- and probiotics have studied synbiotic mixtures. Collectively, studies to date have resulted in inconsistent outcomes in general ICU settings, likely due to the variable effects individual bacterial species exert upon the host [47]. Additionally, the use of synbiotic mixtures is advised against in pancreatitis, where their use was associated with worse outcomes. However, there have been promising results with synbiotic formulations in some patient populations, including transplant [199, 200], major abdominal surgery [201], and trauma patients [202, 203], where treatment reduced overall infection and intestinal permeability. Additionally, certain prebiotic soluble fibers alone may benefit stable critically ill patients with diarrhea by improving *bifidobacteria* levels [204], since low levels of this organism are associated with diarrhea. Due to these discrepancies in general ICU patients, no recommendations are currently given by ASPEN or CCCNG for the use of probiotics, prebiotics, or synbiotics in critically ill patients [42, 47, 48].

Nucleotides

Nucleotides are small molecular weight compounds found in all cells that are used to synthesize RNA and DNA. During rapid cell growth and proliferation nucleotides can become

limiting if *de novo* synthesis and scavenging pathways do not compensate [205, 206]. Under normal conditions, pancreatic enzymes digest dietary RNA from plant and animal food sources and recover approximately 2-5% of ingested RNA [207]. Supplementing nucleotides can reduce the necessity of *de novo* synthesis. Low nucleotide availability can have deleterious effects on immune function, since effective adaptive mediated immune responses require rapid cell proliferation. Mice fed nucleotide-free diets have significantly reduced Th2 immune responses, including antibody production to T lymphocyte-dependent antigen processing [208]. In contrast, nucleotide availability shifts lymphocyte populations towards Th2 cytokine profiles, as opposed to Th1, and enhance macrophage-lymphocyte interactions, which support adaptive immune response [209, 210]. Laboratory studies using enteral nucleotides have also demonstrated beneficial outcomes upon gastrointestinal parameters, including increased villus height, mucosal protein, and brush boarder enzymes [211]. Functionally, nucleotide supplementation limits bacterial translocation and maintains the mucosal barrier in rodent studies [212-214]. No clinical studies have examined the effect of nucleotides alone in critical patients; rather, nucleotides have been added with other nutrition support compounds, including glutamine, arginine, and ω-3 fatty acids. Those studies found no effect upon mortality, but did demonstrate benefits on LOS and infection that were most evident in the malnourished. There are currently no recommendations for the use of nucleotides in critically ill patients.

Appropriate versus Inappropriate Monitors of Nutrition Status

A thorough history and physical examination including recent body weight and dietary changes remains the most important monitor of nutrition therapy. Nutritional status is a balance between pre-existing nutritional status and the degree of hyper metabolism and time until taking

an adequate diet. In the patients undergoing elective general surgery with no inflammation, a low baseline albumin correlates with increased complications when studied in large patient populations [215]. But as the magnitude of surgery increases, the smaller the degree of albumin drop needed to increase complications. Since patient outcome results from the interactions between the extent of pre-existing malnutrition, the magnitude of injury, and the degree and duration of hypermetabolism, several techniques to quantify the degree of stress have been studied, including: measurement of the metabolic rate, assessing the nitrogen losses versus nitrogen intake, the level of serum cytokines, and finally, sequential measurements of serum protein levels. Each technique has a drawback. For example, the metabolic rate is a measurement of oxygen consumption and CO_2 production. Unfortunately, in the most critically ill or injured patients, these measurements are not valid if chest tubes are in place, a high FIO_2 or high levels of PEEP are used during mechanical ventilation, or there exists air leaks around a tracheostomy or an endotracheal tube. Nitrogen balances are flawed in that nitrogen administered is overestimated while nitrogen lost is underestimated. It is almost accurate to quantify nitrogen losses in stool or through an open abdominal wound. Levels of serum cytokines ignore the local production of these individual cytokines and more likely measure spill over from local sites into systemic circulation. For example, in 1992 Ohzata et al, [216] measured IL-6 levels following aortic surgery and noted a peak in IL-6 levels at 24-48 hours which return to normal by 72 hours. However, Balgrie et al, [217] harvested simultaneous splanchnic and systemic samples simultaneously during similar surgeries and showed that portal levels of IL-6 were significantly higher than the systemic levels. The relevance of this is that hepatic protein synthesis is directed by IL-6 to switch from constitutive to acute phase protein production producing elevations in serum fibronectin, CRP, and alpha -1 acid glycoprotein while driving

albumin and pre-albumin levels lower. IL-6 levels within the splanchnic circulation correlate CRP levels, the degree of injury, and the metabolic rate.

We studied sequential serum constitutive and acute phase protein levels on days 1, 4, 7 and 10 in a study of trauma patients randomized to enteral and parenteral feeding [218]. On one day of injury, albumin levels were low and remained low throughout the full 10 day period of study, whether the patients recovered uneventfully or not. Pre-albumin dropped from day 1 measurement to a nadir on day 4 after injury but gradually increased in the two study groups with a more rapid recovery in those receiving enteral feeding. CRP at day 1 was elevated in both groups and increased significantly from baseline in the parenteral fed group but remained relatively stable in the enteral group. Alpha -1 acid glycoprotein was elevated at day 1 and continued to be elevated at days 4, 7 and 10 with essentially no change over time. Thus it appeared that both albumin and alpha -1 acid glycoprotein were unresponsive to recovery while CRP and prealbumin were responsive and could possibly be a monitor of recovery.

We also analyzed combinations of these proteins using published methods, such as the Prognostic Inflammatory and Nutrition Index (PINI) and the Prognostic Nutritional Index. The PINI is a ratio of the acute phase protein (CRP and alpha 1 acid glycoprotein) response to the constitutive protein (pre-albumin and albumin) response. As the PINI increases with increases in acute phase proteins, the risk of complications appears to increase as well. The enteral fed group remained relatively stable in their PINI over time, while parenteral feeding resulted in further PINI increases on days 7 and 10. The Prognostic Nutrition Index (PNI) calculates the risk of complications constitutive protein levels and lymphocyte levels. The higher the PNI, the higher

the risk of complications. Throughout the study, the PNI remained relatively stable and elevated in the both study groups.

In our study, however, we found a lower infection rate in the enterally fed patient compared to the parenteral fed and examined the protein responses relative to the presence or absence of infection by group. As expected, albumin levels in infected patients in both groups were significantly lower than their un-infected cohorts. Surprisingly, albumin levels remained depressed even in uninfected patients for the whole 10 day period of study. Pre-albumin levels dropped by day 4 compared to day 1 in both groups, but recovered in uninfected patients in either group. CRP levels gradually dropped over the 10 day in uninfected patients regardless of route of feeding, but remained elevated in all infected patients. The same response occurred with the PNI. These results confirmed that following injury, serum protein levels albumin and alpha 1 acid glycoprotein are not useful since outcome –good or bad- does not affect them. Sequential pre-albumin and CRP levels appear to discriminate between infected and uninfected patients. These responses however do not reflect nutritional status but rather the presence or absence of complications.

The appropriate monitor for nutritional status in critically ill patients is unknown. Pre-operative albumin may be useful marker in large populations to predict their likelihood of infection, but the relevance to an individual is less precise. A thorough history and physical examination, by an informed clinician, is probably the best predictor of nutritional status.

Conclusions

The concept of 'pharmaconutrition' advocates investigating the effects of individual nutrients upon immune function and clinical outcomes, rather than 'cocktail' formulations. The various dosages, durations, and efficacy of nutrient supplementation make this a extremely difficult and expensive undertaking especially given heterogeneous patient populations and the need for a plethora of treatment arms. However, significant clinical evidence suggests that specific nutrients can improve clinical outcomes when provided to the select patient subpopulations. Updated systematic reviews of the current clinical data and recommendations are available at http://www.nutritioncare.org/library.aspx and http://criticalcarenutrition.com through the American Society for Parenteral and Enteral Nutrition (ASPEN) guidelines and the Canadian Critical Care Nutrition Group (CCCG), respectively.

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