Tumour necrosis factor-α-induced loss of intestinal barrier function requires TNFR1 and TNFR2 signalling in a mouse model of total parenteral nutrition

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Key points

• Total parenteral nutrition (TPN) is critical for patients who cannot tolerate enteral nutrition. However, TPN-associated loss of barrier function leads to an increase in enterically derived pathogens that may harm the patient.
• Tumour necrosis factor-α (TNF-α) is involved in the dysregulation of intestinal barrier function in many animal models.
• The mouse model of TPN provides an excellent, non-destructive approach to examine epithelial barrier dysfunction.
• Tumour necrosis factor-α is shown to be a major mediator of epithelial barrier dysfunction using this TPN model.
• Tumour necrosis factor-α signalling is reliant on both the TNFR1 and TNFR2 pathways to effect epithelial barrier dysfunction.
• Anti-TNF treatment protected against TPN-associated epithelial barrier dysfunction and might prove to be a viable future clinical approach.

Abstract Tumour necrosis factor-α (TNF-α) has been reported to play a central role in intestinal barrier dysfunction in many diseases; however, the precise role of the TNF-α receptors (TNFRs) has not been well defined using in vivo models. Our previous data showed that enteral nutrient deprivation or total parenteral nutrition (TPN) led to a loss of intestinal epithelial barrier function (EBF), with an associated upregulation of TNF-α and TNFR1. In this study, we hypothesized that TNF-α plays an important role in TPN-associated EBF dysfunction. Using a mouse TPN model, we explored the relative roles of TNFR1 vs. TNFR2 in mediating this barrier loss. C57/BL6 mice underwent intravenous cannulation and were given enteral nutrition or TPN for 7 days. Tumour necrosis factor-α receptor knockout (KO) mice, including TNFR1KO, TNFR2KO or TNFR1R2 double KO (DKO), were used. Outcomes included small intestine transepithelial resistance (TER) and tracer permeability, junctional protein zonula occludens-1, occludin, claudins and E-cadherin expression. In order to address the dependence of EBF on TNF-α further, exogenous TNF-α and pharmacological blockade of TNF-α (Etanercept) were also performed. Total parenteral nutrition led to a loss of EBF, and this was almost completely prevented in TNFR1R2DKO mice and partly prevented in TNFR1KO mice but not in TNFR2KO mice. The TPN-associated downregulation of junctional protein expression and junctional assembly was almost completely prevented in the TNFR1R2DKO group. Blockade of TNF-α also prevented dysfunction of the EBF and junctional protein losses in mice undergoing TPN. Administration of TPN upregulated the downstream nuclear factor-κB and myosin light-chain kinase (MLCK) signalling, and these changes were almost completely prevented in TNFR1R2DKO mice, as well as with TNF-α blockade, but not in TNFR1KO or TNFR2KO TPN groups. Tumour necrosis factor-α is a critical factor for...
Introduction

Total parenteral nutrition (TPN), or the removal of all enteral nutrients, is commonly used clinically for patients who cannot tolerate nutrition through their gastrointestinal tract. Despite being life sustaining, clinical usage of TPN has led to an increase in enterically derived pathogens, presumably due to a loss of epithelial barrier function (Buchman et al. 1999). Maintenance of an intact intestinal epithelial barrier is essential in preventing intestinal penetration of luminal toxins, antigens and bacteria. The importance of an intact epithelial barrier function (EBF) has been appreciated by the association of a loss of barrier function with several disease states (Amasheh et al. 2010; Hering et al. 2011; Menard et al. 2012; Schumann et al. 2012). A principal contributor to the regulation of the intestinal EBF is the integrity of the epithelial tight junction ( TJ ) complex, which bridges the interepithelial cell spaces and provides a strong deterrent to the paracellular passage of nutrients, toxins and other intraluminal substances (Mitic & Anderson, 1998; Mitic et al. 2000; Aijaz et al. 2006). Pro-inflammatory signalling clearly plays a critical role in breaking down TJ integrity (Shen et al. 2006; Schwarz et al. 2007; Noth et al. 2011; Cunningham & Turner, 2012; Petecchia et al. 2012; Watson & Hughes, 2012). However, the predominant models used to study loss of EBF have been epithelial injury models, such as inflammatory bowel disease (Amasheh et al. 2009; Arrieta et al. 2009; Edelblum & Turner, 2009; Mankertz et al. 2009; Bereswill et al. 2010). The overt damage to the epithelium in such models can confound the ability to examine the fine interplay of between pro-inflammatory signalling and TJ integrity.

A unique model of EBF loss is the mouse model of enteral nutrient deprivation. In this model, mice are sustained with TPN and have shown a significant loss of EBF without destruction of the epithelium (Sun et al. 2008; Feng et al. 2009). Although the precise mechanisms that drive this EBF loss are not completely known, researchers in our laboratory have previously demonstrated an upregulation of tumour necrosis factor-α (TNF-α) and expression of its receptor TNFR1 within the small bowel mucosa in this TPN model. These pro-inflammatory changes were associated with a decline in intestinal epithelial cell (IEC) proliferation and an increase in IEC apoptosis (Feng & Teitelbaum, 2012). Tumour necrosis factor-α has a central role in many diseases associated with intestinal epithelial barrier dysfunction, including inflammatory bowel disease (Baert & Rutgeerts, 1999; Baert & Rutgeerts, 2000; Suenaert et al. 2002), intestinal ischaemia (Tamion et al. 1997; Taylor et al. 1997) and graft-versus-host disease (Brown et al. 1999). Over the past two decades, a deeper understanding of the mechanisms that lead to a TNF-α-induced loss of EBF has been gained. Tumour necrosis factor-α, along with other pro-inflammatory mediators, such as LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells) and interleukin-1β (Schwarz et al. 2007; Al-Sadi et al. 2008, 2010), help to mediate this loss of EBF. Cytokine signalling leads to phosphorylation of myosin light chain (MLC) and a loss of zonula occludens-1 (ZO-1), as well as an internalization of occludin within the cytoplasm via incorporation into caveolin-1-containing vesicles (Marchiando et al. 2010; Cunningham & Turner, 2012). Previous work using in vitro models has suggested that this signalling pathway is dependent on a TNFR2 pathway (Wang et al. 2005). This is intriguing, and if this pathway is the major mediator of loss of EBF it may be an important target for future clinical therapies in conditions which are associated with a loss of EBF, such as inflammatory bowel disease. However, the in vitro approach of these investigations may not fully represent modulation of EBF within an in vivo setting.

We hypothesized that the increased expression of TNF-α is a critical factor which contributes to TPN-associated loss of intestinal barrier function and that TNF-α receptor ligation plays an important role in this process. Our present study demonstrated a critical role of TNF-α signalling in the mechanisms which drive the loss of
Intestinal barrier function in total parenteral nutrition

EBF in this mouse model of TPN. Using TNFR1R2 double knockout (DKO) and TNFR1 or TNFR2 single knockout (KO) mice, this work demonstrates the novel finding that both the TNFR1 and the TNFR2 signalling pathways are critical for mediation of the loss of EBF in this model. Blockade of TNF-α signalling was effective at protecting EBF. These findings are important in that this improved understanding of EBF loss may help to direct future therapeutic options for patients dependent on TPN as well as those who suffer from a loss of EBF from other disease processes, such as inflammatory bowel disease.

Methods

Animal model of parenteral nutrition

Male C57BL/6j specific pathogen-free mice (10–12 weeks old; Jackson Laboratory, Bar Harbor, ME, USA) were maintained in temperature-, humidity- and light-controlled conditions. Mice were initially fed ad libitum with standard mouse chow and water and were allowed to acclimate for 1 week prior to surgery. During the administration of intravenous solutions, mice were housed in metabolic cages to prevent coprophagia. Studies conformed to guidelines for care established by the University Committee on Use and Care of Animals at the University of Michigan, and protocols were approved by that committee (no. 03986).

Methods of cannulation and TPN infusion were identical to those previously described (Kiristioglu & Teitelbaum, 1998; Wildhaber et al. 2003; Yang et al. 2007). The control group received intravenous crystalloid solution at 0.2 ml h⁻¹ and standard laboratory mouse chow. The TPN groups received intravenous TPN solution at 4.8 ml (24 h)⁻¹. All animals were killed at 7 days using CO₂.

Male TNFR1R2DKO (Tnfrsf1atm1Imx Tnfrsf1btm1-Imx/J), TNFR1KO (Tnfrsf1atm1Imx/J) and TNFR2KO (Tnfrsf1btm1Mwm/J) mice were used in this study. All mice had a C57BL6 background (Jackson Laboratories); body weight and age were matched to wild-type (WT) mice (Feng & Teitelbaum, 2012). In some experiments, both WT control and TPN groups were injected intravenously with recombinant mouse TNF-α (rmTNF, 200 ng (g body weight)⁻¹; ImmunoTools, Friesoythe, Germany) 3 h before they were killed (Günther et al. 2011). In another group of TPN and enterally fed groups, WT mice were given 200 μg (kg body weight)⁻¹ of Etanercept (Immunex Corporation, Thousand Oaks, CA, USA) subcutaneously every 48 h for three doses, and another dose of 500 μg (kg body weight)⁻¹ was given 3 h before they were killed (Fries et al. 2008; Nemoto et al. 2011).

Intestinal epithelial resistance and permeability measurements

Ussing chambers (Physiologic Instruments, Inc., San Diego, CA, USA) were used to assess full-thickness proximal small intestinal (6 cm from the ligament of Treitz) barrier function (Yang et al. 2002). Transepithelial resistance (TER) was measured after a 3 min equilibration and determined using Ohm’s Law (Reims et al. 2006). Permeability of the small intestine was assessed with fluorescein isothiocyanate (FITC)–dextran (4000 kDa at 50 mg ml⁻¹; Sigma-Aldrich) as a tracer molecule (Bücke et al. 2011). For this, 150 μl FITC–dextran was added to the mucosal compartment of Ussing chambers after 30 min equilibration, and 500 μl of buffer was taken from the serosal compartment 2 h after incubation for analysis the FITC–dextran concentration. Fluorescence was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) at an excitation wavelength of 492 nm and an emission wavelength of 515 nm. Permeability was expressed as the mucosal-to-serosal clearance of FITC–dextran-4000 (Bücke et al. 2011).

Western blotting

For protein analysis, IECs were isolated from the small intestine using a modification of Grossmann’s technique. Intestinal epithelial cells were used to prepare protein lysates for Western blotting; techniques were as previously described (Grossmann et al. 1998; Schwarz et al. 2007). The purity of the epithelial isolates was assessed by microscopic inspection at isolation and Western blot analysis for E-cadherin and smooth muscle actin (not shown); the epithelial isolates consisted primarily of intact released crypts that were E-cadherin positive and smooth muscle actin negative. Blots were stripped and reprobed with monoclonal mouse anti-β-actin antibody to confirm equal loading of protein. The results are expressed as the ratio of relative protein expression to β-actin expression. The antibodies used for immunoblots were as follows: rabbit anti-ZO-1 and mouse anti-occludin (Life Technologies Corporation, Carlsbad, CA, USA); mouse anti-E-cadherin (BD, San Jose, CA, USA); mouse anti-κBζ and mouse anti-phosphate MLC (p-MLC; Cell Signalling, Danvers, MA, USA); and mouse anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibody was either goat anti-mouse-HRP or goat anti-rabbit-HRP (Santa Cruz).

Immunofluorescence microscopy

A 0.5 cm section of fresh jejunum (~10 cm from the ligament of Treitz) was harvested, and immunofluorescence staining was performed as previously
described (Feng et al. 2009; Nose et al. 2010). Fluorescence was analysed by using an Olympus BX-51 upright light and fluorescence microscope. Images were stacked using Nikon image software and processed using Adobe Photoshop CS4.

**Data analysis**

Data were expressed as means ± SD. Statistical analysis employed Student’s paired t tests for comparison of two means, a one-way ANOVA for comparison of multiple groups (with a Bonferroni post hoc analysis to assess statistical differences between groups) and a two-way ANOVA for categorical data (Prism software; GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was defined as $P < 0.05$.

**Results**

**Total parenteral nutrition-associated barrier dysfunction is dependent on TNF-α receptor signalling**

In order to begin to address the dependence of TNF-α receptors on TPN-associated loss of EBF, a series of TNFR1R2DKO and TNFR1 or TNFR2 single KO mice were used. It has been demonstrated previously that enteral nutrient deprivation leads to a loss of EBF (Yang et al. 2009; Nose et al. 2010; Hagiwara et al. 2011; Feng et al. 2012). This present study confirmed that after 7 days of TPN, TER was decreased 1.6-fold compared with enteral controls (Fig. 1A). Additionally, permeation of FITC–dextran to the serosal side increased 7-fold vs. controls (Fig. 1B).

Transepithelial resistance was then examined in TNF receptor KO mice. Interestingly, the use of TNFR1KO resulted in partial prevention of the loss of TER (58.6% of enteral control mice), whereas TNFR2KO mice had no impact on the restoration of TER. However, use of TNFR1R2DKO mice further improved TER compared with WT TPN mice (72.7% of control levels), and TER rose significantly above that of TNFR1KO mice (14.1% further improvement in TER). Examination of FITC–dextran permeability showed similar results, with some important distinctions. Both TNFR2KO and TNFR1KO mice given TPN had a significant 50 and 61% reduction (respectively) in permeation compared with wild-type mice. Importantly, the use of TNFR1R2DKO mice led to a complete restoration of permeability levels, which were essentially the same as those of enterally fed mice (Fig. 1B). This suggests that both the TNFR1 and TNFR2 signalling pathways have important roles in mediating EBF loss, and this was explored further. When examining TER and FITC–dextran permeation in all control groups, there was no difference between wild-type mice and other strains (data not shown).

**Figure 1. Total parenteral nutrition (TPN)-associated barrier dysfunction is dependent on integrity of receptors TNFR1 and TNFR2**

A, transepithelial resistance (TER) measurements were performed in Ussing chambers. The results are the mean of a minimum of 6 mice per group over 90 min, preceded by a 30 min equilibration period. Note that TPN administration led to a 50% decrease in TER vs. controls in wild-type (WT) mice. This decline was partly prevented in TNFR1 knockout (KO) mice, but only slightly in TNFR2KO mice. However, TNFR1R2 double knockout (DKO) mice showed significant prevention of the reduction in TERs. B, paracellular permeability was measured by the mucosal to serosal passage of FITC–dextran. Administration of TPN led to a marked increase in FITC–dextran permeation in WT mice. This increase in permeability was partly prevented in either TNFR1KO or TNFR2KO TPN mice and almost completely prevented in TNFR1R2DKO mice. *$P < 0.05$, **$P < 0.01$. © 2013 The Authors. The Journal of Physiology © 2013 The Physiological Society
Total parenteral nutrition-associated loss of tight junction and adherence junction protein expression was prevented in TNFR1R2DKO mice

The tight junction proteins ZO-1 and occludin were measured with immunoblotting and immunofluorescence staining. Similar to our previous publication (Sun et al. 2008; Nose et al. 2010), expression of ZO-1 and occludin protein decreased significantly with administration of TPN (Fig. 2A and B). The effect of TNFR1 and TNFR2 on the abundance of these tight junction proteins was then investigated. The results of Western blots showed that ZO-1 and occludin remained markedly decreased in TNFR2KO, and amounts were not significantly greater than WT TPN levels. Use of TNFR1KO TPN mice partly prevented the loss of these junctional proteins; however, use of TNFR1R2DKO TPN mice completely prevented the loss of these proteins (Fig. 2A and B).

Immunofluorescence staining showed a clear and sharp expression of ZO-1 and occludin along the surface of

![Figure 2. Deletion of both TNFR1 and TNFR2 prevents TPN-associated tight junction protein down-regulation](image)

Expression of zonula occludens-1 (ZO-1) and occludin was measured with Western immunoblots and immunofluorescence staining. The mean ratios of these proteins to β-actin are reported for a minimum of at least \( n = 6 \) per group. \( \ast \ast P < 0.01, \ast P < 0.05 \). A, representative example of immunoblots and relative density to β-actin. B, ZO-1 and occludin protein levels declined with TPN, and this decline was totally prevented in TNFR1R2DKO TPN mice. A partial prevention was seen in TNFR1KO TPN mice, but no change was observed in the TNFR2KO TPN group. C, immunofluorescence staining showed decreased ZO-1 (red) and occludin expression (green) with TPN. This was almost completely prevented in TNFR1R2DKO mice (Ce, g and m) and partly prevented in TNFR1KO mice (Cf, i and n), but was not changed in TNFR2KO mice (Ce, j and o). D, High-magnification images showing that the co-localization of ZO-1 and occludin (yellow, Da) was lost in WT TPN mice (Db), with an internalization of occludin (*). This co-localization was nearly completely recovered in TNFR1R2DKO mice (Dc). Diffuse ZO-1 expression and occludin internalization persisted in TNFR1KO mice (Dd); and use of TNFR2KO mice produced similar results to those for WT TPN mice.
epithelial cells in enterally fed mice (Fig. 2Ca and f), which was lost with enteral deprivation (Fig. 2Cb and g). The co-localization of these two proteins was found in the enterally fed group, but was lost in TPN mice (Fig. 2Da and b). Administration of TPN resulted in disassembly of ZO-1 and occludin, along with an internalization of occludin within the cytoplasm (asterisks in Fig. 2Db), consistent with other models where TNF-α is given exogenously (Shifflett et al. 2005; Clayburgh et al. 2006; Tang et al. 2010). Use of TNFR1KO or TNFR2KO mice did not prevent this loss. However, the loss of intensity of ZO-1 along the epithelial surface was partly prevented in TNFR1KO TPN mice. Use of TNFR1R2DKO TPN mice resulted in a virtually complete prevention of the loss of ZO-1 and occludin expression at the tight junction complex. At high magnification (Fig. 2D), occludin expression, TJ assembly and co-localization of these two proteins was protected in TNFR1R2DKO TPN mice (Fig. 2D, III). Zonula occludens-1 expression and localization within the tight junction complex was partly recovered in TNFR1KO TPN mice. However, ZO-1 was more diffusely expressed along the epithelial surface, and an internalization of occludin was still observed in TNFR1KO TPN mice (Fig. 2Dd). Total parenteral nutrition given to TNFR2KO mice failed to prevent the loss of ZO-1 and occludin expression and junctional disassembly (Fig. 2De).

Thus, although TNFR2KO mice failed to show an improvement in TER and TJ integrity, an internalization of TJs and loss of TER persisted until both TNFR1 and TNFR2 were eliminated, demonstrating the importance of both the TNF-α signalling pathways.

The expression levels of claudin-1, -2 and -4 were measured with Western immunoblots. Administration of TPN led to a significant downregulation in claudin-1 expression (2-fold vs. enteral controls). This decline was prevented in TNFR1R2DKO and TNFR1KO mice, but not in TNFR2KO mice (Fig. 3A and B). Total parenteral nutrition did not obviously affect the protein expression of claudin-2 in WT mice. Interestingly, claudin-2 expression was upregulated 2.5-fold above enteral controls in the TNFR1R2DKO TPN group; however, claudin-2 expression was not affected in TNFR1 or TNFR2 single KO TPN groups (Fig. 3A and C). Total parenteral nutrition-associated downregulation of claudin-4 expression was decreased in WT TPN mice, and expression was somewhat reduced with the use of TNFR1R2DKO mice.

Zonula occludens-1 acts as a functional cross-linker between the E-cadherin–catenin complex and the actin-based cytoskeleton (Itoh et al. 1997; Shen, 2012). We have previously reported (Feng et al. 2009) that the abundance of E-cadherin protein was significantly decreased with TPN in WT mice. In the present study, the decrease in E-cadherin protein expression assessed by immunoblotting was totally prevented in TNFR1R2DKO TPN mice; however, the use of TNFR1 or TNFR2 single KO mice did not affect the loss of E-cadherin expression (Fig. 4A and B).

Co-localization of ZO-1 and E-cadherin was also measured with immunofluorescence staining. E-Cadherin was expressed along the surface and between IECs in the control group; furthermore, a tight co-localization of ZO-1 and E-cadherin was observed at the IEC apical surface (Fig. 4Ca and f). The expression levels of both ZO-1 and E-cadherin were markedly decreased in WT TPN mice, and TPN also led to a loss of co-localization of these proteins (Fig. 4Cb and g). Use of TNFR1R2DKO mice prevented this TPN-associated loss of E-cadherin expression and maintained ZO-1 and E-cadherin co-localization (Fig. 4Cc and h). However, the use of single TNFR1KO (Fig. 4Cd and i) or TNFR2KO mice (Fig. 4Ce and j) failed to protect E-cadherin expression and assembly in TPN mice. Thus, the loss of the adherence junction integrity appears to be dependent on TNFR1 and TNFR2 signalling.

**Total parenteral nutrition-associated upregulation of myosin light chain activation and nuclear factor-κB (NF-κB) signalling is TNF receptor dependent**

Activation of the myosin light chain pathway is well known to play a critical role in tight junction regulation in intestinal (Clayburgh et al. 2005; Ye et al. 2006) and lung epithelial monolayers (Dull et al. 2003). In addition, in inflammatory bowel disease models (Su et al. 2009; Weber et al. 2010; Gilbert et al. 2012), the activation of MLC via its phosphorylation has been shown to be responsible for the internalization of junctional proteins, including occludin, and is one of the key mechanisms for the loss of barrier function. Phosphorylated MLC (p-MLC) expression (measured via immunoblots) showed that TPN led to an upregulation of p-MLC expression by 3-fold vs. controls (Fig. 5A and B). Interestingly, this upregulation was totally prevented in TNFR1R2DKO TPN mice. However, both TNFR1KO and TNFR2KO TPN mice maintained high expression of p-MLC, demonstrating the importance that both receptors have in driving this mechanism of EBF loss.

Activation of NF-κB in IECs can lead to production of pro-inflammatory cytokines and chemokines that secondarily recruit immunocytes, thereby initiating an inflammatory amplification cascade (Barnes & Karin, 1997). Nuclear factor-κB is regulated by its release from activated (phosphorylated) by IkB kinase (IKK) complex consisting of the IKBa and IKBβ catalytic subunits.
and the IKKγ/NEMO regulatory subunit (DiDonato et al. 1997; Rothwarf & Karin, 1999; Ghosh & Karin, 2002). To examine activation of NF-κB, IKBα expression was measured with immunoblotting (Fig. 5A and C). Total parenteral nutrition significantly decreased IKBα protein expression (3.8-fold vs. WT controls), indicating TPN-driven NF-κB activation. Expression IKBα protein was totally prevented in TNFR1R2DKO TPN mice, and levels returned to control values. A partial prevention of the decline in IKBα was seen in the TNFR1KO and TNFR2KO TPN mice.

**Anti-tumour necrosis factor treatment prevented TPN-associated loss of epithelial barrier function, and exogenous TNF-α further impaired barrier function**

Etanercept, an engineered TNFR2 protein that acts as a TNF-α antagonist by competitively binding soluble TNF-α, was used to study the effect of blocking endogenous expression of TNF-α. Etanercept treatment partly prevented the TPN-associated decline in small bowel TER (17 ± 2.12 vs. 12.6 ± 0.89 Ω cm², TPN + Etanercept vs. TPN + isotype IgG control; P < 0.01; Fig. 6A).

**Figure 3. Claudin abundance is regulated by TNFR1 and TNFR2 signalling in mice receiving TPN**

The expression levels of claudins-1, -2 and -4 were measured with Western blotting in each group (n ≥ 6 per group). A, representative immunoblots and density expressed relative to that of β-actin. B–D, abundance of claudin-1, -2 and -4 proteins in each study group. Claudins-1 and -4 declined in TPN mice. The decline in claudin-1 was partly prevented in TNFR1KO mice, and both claudin-1 and claudin-4 were restored to nearly the same levels as the control values in TNFR1R2DKO. Note that claudin-2 expression was not affected by TPN, but was markedly upregulated in TNFR1R2DKO TPN mice. *P < 0.05, **P < 0.01.
Examination of permeability also showed that Etanercept treatment significantly decreased mucosal to serosal passage of FITC–dextran (8.79 ± 1.29 vs. 13.18 ± 3.22 μg ml⁻¹). TPN + Etanercept vs. TPN + isotype IgG control; *P < 0.001; Fig. 6B). Etanercept treatment did not affect TER and permeability in enteral-fed groups (data not shown). Zonula occludens-1, occludin and E-cadherin expression were significantly increased after Etanercept treatment vs. isotype IgG treatment in TPN mice (Fig. 6C and D). Furthermore, immunofluorescence staining showed a partial prevention in the loss of co-localization and distribution of ZO-1/occludin and ZO-1/E-cadherin with Etanercept treatment (Fig. 6E and F). Etanercept treatment also partly prevented the TPN-associated increase in NF-κB and MLCK activation, bringing p-MLC and IKBα expression close to control levels (Fig. 6C and D).

In order to examine the effect of TNF-α further in this TPN model, exogenous recombinant mouse TNF-α (rmTNF) was injected intravenously in WT control and TPN mice 3 h before the mice were killed. The TER values decreased significantly after rmTNF injection. Transepithelial resistance declined to 16.9 ± 4.8 Ω cm⁻² in the enteral-fed control group with rmTNF treatment compared with 26.5 ± 2.7 Ω cm⁻² in control mice given saline (*P < 0.001; Fig. 7A). In addition, TER decreased to 5.35 ± 0.73 Ω cm⁻² in WT TPN + rmTNF mice, which was significantly lower than the TER seen in WT TPN + saline mice (13 ± 1.19 Ω cm⁻²). This trend was also seen with permeability data. Serosal FITC–dextran concentration increased (8.42 ± 1.48 μg ml⁻¹) in control mice treated with rmTNF vs. saline (2.25 ± 0.94 μg ml⁻¹; *P < 0.001). In the TPN group, rmTNF further increased FITC–dextran concentration (19.41 ± 8.41 μg ml⁻¹) compared with 16.59 ± 5.14 μg ml⁻¹ in TPN mice without rmTNF), but the difference was not significant (Fig. 7B). Zonula occludens-1 and occludin expression measured by immunoblotting were not affected in control and TPN groups after rmTNF (Fig. 7C). However, compared with saline injection, immunofluorescence staining showed that the assembly of ZO-1, occludin and E-cadherin was markedly deranged with rmTNF injection to a greater degree than in WT TPN mice (Fig. 7D and E). In enteral controls, exogenous rmTNF disrupted ZO-1 and occludin distribution (Fig. 7Da and c) and also disrupted the co-localization of ZO-1 with E-cadherin (Fig. 7Ea and c). Furthermore, exogenous rmTNF injection totally abolished ZO-1 and occludin expression, and led to a further decline in E-cadherin expression, resulting in a markedly disrupted TJ and adherens junction assembly in TPN mice (Fig. 7Da and d, Fig. 7Ea and d).

**Figure 4. Both TNFR1 and TNFR2 contribute to the TPN-associated E-cadherin downregulation**

E-Cadherin expression was measured in isolated intestinal epithelial cells (IECs) using immunoblotting and immunofluorescence staining. **P < 0.05, *P < 0.01. A, representative Western blots and density expressed relative to β-actin. B, TPN led to a loss of E-cadherin expression. This was completely prevented in TNFR1R2DKO mice, but not in TNFR1 or TNFR2 single KO mice. C, immunofluorescence staining showed that TPN administration led to a marked loss of E-cadherin (green) expression (Cb and g) and a loss of E-cadherin and ZO-1 (red) co-localization (yellow). This change was totally prevented in TNFR1R2DKO TPN mice (Cc), and co-localization of E-cadherin with ZO-1 was recovered (Ch). Neither TNFR1KO nor TNFR2KO mice could prevent these TPN-associated changes (Cd, e, i and j).
Discussion

Total parenteral nutrition is vital for the support of patients who cannot tolerate enteral nutrition. It is estimated that over 350,000 patients yearly in the USA receive TPN, resulting in over 11.5 million patient days of care each year; a number which has almost doubled in the past 20 years (HCUPnet, 2010). Unfortunately, TPN results in both morphological and functional changes to the intestinal epithelium, including intestinal epithelial cell apoptosis, loss of IEC proliferation and a loss of EBF (Feng et al. 2009; Feng & Teitelbaum, 2012). Total parenteral nutrition-associated barrier dysfunction has been reported in humans and rodent models (Buchman et al. 1995; Kansagra et al. 2003; Sun et al. 2008; Nose et al. 2010), and may result in endotoxins and even bacteria entering the systemic circulation.

The formation and maintenance of an intact intestinal barrier is critical to maintain normal physiological function and prevent diseases, such as ulcerative colitis, Crohn disease, chronic diarrhoeal disease and other infectious and inflammatory intestinal diseases (Meddings et al. 1994). Significant data suggest that TNF-α is involved in the dysregulation of intestinal EBF (Lorenzo-Zúñiga et al. 2006; Kolodziej et al. 2011; Marchiando et al. 2011; Noth et al. 2011; Scharl et al. 2011; Ye et al. 2011) and is a critical pathogenic component of many related diseases. Tumour necrosis factor-α and other pro-inflammatory factors, such as interferon-γ, are major factors which contribute to TPN-associated loss of EBF. An upregulation of small intestinal TNF-α and TNFR1 expression was identified in our TPN mouse model and was closely linked with loss of IEC proliferation and an increase in IEC apoptosis (Feng & Teitelbaum, 2012). Researchers in our laboratory previously reported that TPN-associated loss of small intestinal EBF was partly prevented using interferon-γ KO mice; however, the role of TNF-α has not been investigated. Importantly, this TPN model is a very useful tool to examine the role of the TNF-α signalling pathway in the loss of intestinal EBF, as well as the mechanisms and signalling pathways used by TNF-α. Unlike other models, which rely on a mucosal injury, such as chemical-induced mucosal injury or exogenous TNF-α (Clayburgh et al. 2005), the TPN model offers a unique

Figure 5. Total parenteral nutrition-associated upregulation of nuclear factor-κB (NF-κB) and myosin light chain signalling is TNFR1R2 dependent in vivo. The expression levels of phosphorylated myosin light chain (p-MLC) and the NF-κB inhibitory regulator IκBα were measured in IEC isolates with Western blotting. *P < 0.05, **P < 0.01.
approach to study the effects of these pro-inflammatory cytokines in a very clinically relevant setting. Importantly, while TPN administration did lead to mucosal atrophy, due to epithelial cell apoptosis and loss of proliferation, it was not associated with any major erosions of the epithelial lining. In order to understand better how TPN-associated EBF loss occurs, we sought to determine whether one or both TNF receptors were necessary for TNF-induced barrier dysfunction. In the present study, we showed that TPN-associated loss of EBF was dependent on both TNFR1 and TNFR2, and that without blocking both signalling pathways EBF loss was incompletely prevented. This finding shows, for the first time, that both TNFR1 and TNFR2 signalling play an important role in mediating intestinal epithelial barrier dysfunction.

In inflammatory bowel disease, modulation of intestinal epithelial MLCK expression and its activation of MLC to phospho-MLC play a critical role in the mechanisms that result in EBF loss (Chen et al. 2012; Cunningham & Turner, 2012; Gilbert et al. 2012). Our study showed that TPN led to increased p-MLC expression in IECs and that this was totally prevented in TNFR1R2DKO mice, but not in

Figure 6. Etanercept treatment prevents TPN-associated loss of epithelial barrier function
Wild-type TPN mice were given 200 μg (kg body weight)⁻¹ of the tumour necrosis factor-α (TNF-α) antagonist Etanercept subcutaneously, every 48 h for three doses, with another dose of 500 μg kg⁻¹ given 3 h before the mice were killed. Controls were WT TPN mice given isotype IgG. *P < 0.05, **P < 0.01. A, Etanercept prevented the TPN-associated decline in TER. B, Etanercept also prevented the TPN-associated increase in permeability to FITC-dextran. C, representative Western blots from isolated IECs. D, protein abundance of ZO-1, occludin and E-cadherin measured by immunoblots relative to β-actin. The junctional protein abundance showed that Etanercept treatment prevented loss of the junctional proteins. Etanercept also prevented the rise in p-MLC and decline in IKBα. E and F, ZO-1, occludin and E-cadherin immunofluorescence staining. Prevention by Etanercept of the loss of junctional proteins is also seen by immunofluorescence staining. Note also the preservation of both ZO-1/occludin and ZO-1/E-cadherin co-localization (white arrows).
TNFR1 or TNFR2 single KO TPN mice. This demonstrates a critical need for both TNFR1 and TNFR2 signalling to mediate this downstream p-MLC expression in vivo.

Nuclear factor-κB is a major downstream signalling factor following TNF-α signalling, and activation of NF-κB could be one of the mechanism leading to increased epithelial permeability (Kisseleva et al. 2006; Chen et al. 2008) and tight junction dysfunction (Wachtel et al. 2001). Recently, NF-κB binding motifs were demonstrated to be present on the MLCK promoter region, and these served as a regulatory site to induce MLCK gene activation (Ye & Ma, 2008). In the present study, we showed that TPN administration resulted in an upregulation of NF-κB activation, and that blockade of either TNFR1 or TNFR2 was insufficient to prevent NF-κB activation. Activation of NF-κB was completely blocked only in TNFR1R2DKO TPN mice or with the use of Entanercept. This further emphasizes the importance of both TNF signalling pathways in driving NF-κB activation and MLCK signalling in this model.

The claudins are a large family of transmembrane adhesion proteins located at intercellular tight junctions (Zeissig et al. 2007). A considerable number of studies have demonstrated that claudins can modulate TER through regulation of ionic charge selectivity (Van Itallie et al. 2001; Amashe et al. 2002; Colegio et al. 2002; Spring, 2002), providing convincing evidence that claudins line the paracellular pores. Claudin-1 and claudin-4 provide strength to the epithelial barrier, and both were markedly downregulated in TPN mice; and this loss was partly prevented in TNFR1R2DKO mice. Claudin-2 has been identified as an electrically ‘leaky’ claudin (Furuse et al. 2001; Amashe et al. 2002; Holmes et al. 2006) and is concentrated in intestinal crypts (Holmes et al. 2006), where larger paracellular pores have been reported (Marcial et al. 1984). Administration of TPN did not affect claudin-2 expression. In fact, surprisingly, a higher expression of claudin-2 was observed in TNFR1R2DKO TPN mice compared with the other groups. As available data suggest that it is the pattern of claudin isoform expression that defines tight junction charge selectivity (Simon et al. 1999; Van Itallie et al. 2001), TNF-α may act by globally removing claudins from the tight junction complex, and not by altering the expression of specific claudin isoforms. It is possible that in the absence of TNF-α signalling in TNFR1R2DKO TPN mice, a normally counter-regulatory mechanism between TNF-α signalling and claudin-2 may be disrupted.

Anti-tumour necrosis factor treatment was used as an alternative approach to block TNF-α signalling. Use of Entanercept, a modified TNFR2 that binds competitively to circulating TNF-α, partly prevented TPN-associated barrier loss. Conversely, exogenous TNF-α treatment led to marked exacerbation in IEC barrier dysfunction in both control and TPN mice (greater than that of untreated TPN mice). These findings further demonstrate that TNF-α signalling plays a critical role in the TPN-associated IEC barrier dysfunction. However, neither TNFR1R2DKO nor anti-TNF treatment completely prevented barrier dysfunction in TPN mice. This suggests that TNF-α is most likely not to be the only factor involved in this mechanism. Clearly, the actions of other pro-inflammatory cytokines that are expressed in excess during TPN administration should be considered. It has

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**Figure 7. Administration of TNF-α increased the loss of barrier function in wild-type mice receiving TPN**

Wild-type enterally fed (control) mice or mice receiving TPN were treated with either saline or recombinant mouse TNF-α (rmTNF, 200 ng (g body weight)^−1^) given i.v. 3 h before the end of the 7 day study period. *P < 0.05, **P < 0.01. A, note a further decline in TER with exogenous TNF-α. B, FITC-dextran showed a further increase in permeability with TNF-α. C, Western blots. D and E, immunofluorescence staining showed that rmTNF-α treatment led to a further loss in ZO-1, occludin and E-cadherin staining intensity for both control and TPN groups.
been reported that interferon-γ has an important role in modulating gut barrier function, which could contribute to TPN-associated barrier dysfunction either dependent on or independent of TNF-α signalling. Also, interleukin-6 expression was shown to be upregulated in our TPN model (Wildhaber et al. 2005), and this cytokine can also negatively modulate intestinal tight junction function (Suzuki et al. 2011; Jonker et al. 2012). Therefore, a further exploration of the cross-talk among these cytokines will be needed in future studies.

Although using a distinct different approach, these findings were different from those previously reported (Wang et al. 2006). In the detailed report by Wang et al. (2006), interferon-γ priming increased TNFR2 expression and resulted in loss of EBF. Furthermore, this action was independent of the TNFR1 signalling pathway. The distinction between those findings and the present report may be due to the fact that our study used an in vivo model, whereas their study used an in vitro approach. The TPN model allows for a variety of other factors that may be interdependent on an intact TNF-α signalling pathway. For example, TNF-α and ErbB signalling demonstrate a tight interdependence (Yamaoka et al. 2008). In our TPN mouse model, TNF-α and ErbB signalling were markedly unbalanced, with an increase in TNF-α and loss of ErbB signalling (Feng et al. 2011). This loss in ErbB signalling is a key factor associated with loss of IEC proliferation and increased IEC apoptosis, and was prevented in the present report with the use of TNFR1R2DKO mice.

One of the shortcomings of the present work is that we used whole-animal knockouts (TNFR1 and TNFR2) for our studies. Thus, we have not fully dissected out the potential interaction of TNFR signalling between the myeloid and epithelial cell populations. Unfortunately, there are no epithelial specific knockouts readily available, but future studies could address this with the development of such mice or with the use of bone marrow chimera studies. One of the other limitations of the present study was the fact that we are using a single strain of mice, C57BL/6. While we have studied this strain extensively, we do acknowledge that the use of other mouse strains, which have different immune phenotypes, might yield potentially different responses. Finally, administration of TPN leads to mucosal atrophy, due to IEC apoptosis (Feng & Teitelbaum, 2012). It is possible that apoptosis itself might be a contributory factor to the loss of EBF.

In conclusion, our study demonstrated that TNF-α is a critical factor for TPN-associated epithelial barrier dysfunction. Importantly, we demonstrated that both TNFR1 and TNFR2 receptors were involved in the loss of EBF. Furthermore, both MLCK and NF-κB signalling appear to be important downstream mediators involved in this TNF-α signalling process. The importance of these findings may extend well beyond this TPN model and may help to guide future therapies for a number of inflammatory processes in the gastrointestinal tract.

References


Intestinal barrier function in total parenteral nutrition


