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# Lipopolysaccharide Exposure is Linked to Activation of the Acute Phase Response and Growth Failure in Pediatric Crohn's Disease and Murine Colitis

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## Abstract

**Background—**Systemic exposure to lipopolysaccharide (LPS) has been linked to clinical disease activity in adults with IBD. We **hypothesized** that markers of LPS exposure and the acute phase response (APR) would be increased in pediatric IBD patients with growth failure, and that LPS signaling would be required for induction of the APR in murine colitis.

**Methods—**Serum markers of LPS exposure, endotoxin core IgA antibody (EndoCAb), and the APR, LPS binding protein (LBP), were quantified in pediatric IBD patients and controls. LBP and cytokine production were determined after administration of trinitrobenzene sulfonic acid (TNBS) enemas to mice with genetic deletion of Toll-Like receptor 4 (TLR4), and wild type (WT) controls.

**Results—**Serum EndoCAb and LBP were significantly elevated in patients with Crohn's disease (CD), compared to disease controls with Ulcerative Colitis (UC) and healthy controls ($p<0.001$). This was independent of disease activity or location. CD patients with elevated serum EndoCAb and LBP exhibited linear growth failure which persisted during therapy. Serum LBP increased in WT mice following TNBS administration, in conjunction with increased serum TNF-α, IL-6, and IL-10, and expansion of regulatory T cell numbers. Both the APR and expansion of foxp3+ T cells were abrogated in TLR4 deficient mice, in conjunction with a reduction in acute weight loss.

**Conclusions—**LPS exposure and a persistent APR are associated with growth failure in pediatric CD. LPS signaling is required for the APR in murine colitis. Therapies targeting this pathway may benefit the subset of patients with refractory growth failure.

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**Keywords**

acute phase response; endotoxin; inflammatory bowel disease; linear growth

## INTRODUCTION

The Inflammatory Bowel Diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD), are chronic relapsing and remitting disorders of the gastrointestinal tract. The pathogenesis of IBD is complex and multi-factorial. Current evidence suggests that IBD develops in individuals with a genetic predisposition to respond aberrantly to the enteric flora, leading to an expansion of pro-inflammatory T-cells in the gut (1). In support of this, genetically susceptible mice raised in germ free conditions do not develop colitis until colonization with commensal bacteria (2). Recent studies in human IBD have focused on the luminal flora as a key environmental trigger. Specifically, these studies have implicated genotypic variation in microbial pattern recognition (*NOD2* and *TLR4*) and autophagy (*ATG16L1* and *IRGM*) in susceptibility (3,4). Moreover, CD patients with increased sero-reactivity to microbial products have been shown to experience a more aggressive course complicated by increased rates of stricturing/internal penetrating behavior (5). While linear growth failure is an important complication of pediatric CD, an association with increased exposure to microbial products has not been explored.

Considering the preponderance of gram-negative bacteria in the gastrointestinal tract, the role of endotoxin (lipopolysaccharide; LPS) in activation of immunity and complications of disease in patients with IBD is important to elucidate. Increased translocation across a defective mucosal barrier is a prevailing hypothesis in the etiology of IBD (6). Previous studies have identified circulating endotoxin in adults with IBD and these endotoxin levels have correlated with disease activity, while therapeutic reductions in endotoxin resulted in symptomatic improvement (7–9). Due to its short half-life and frequent contamination of environmental surfaces, endotoxin is difficult to measure in serum; therefore, several studies have utilized surrogate markers for endotoxin exposure. Lipopoysaccharide binding protein (LBP) is an acute phase protein produced by hepatocytes and paneth cells in response to both LPS and cytokines (10). LBP catalyzes delivery of LPS monomers to membrane-bound CD14, or to soluble CD14 (sCD14), which, in turn, delivers LPS to myeloid differentiation protein-2 (MD-2). LPS/MD-2 heterodimers represent the activating ligand for the signaling LPS receptor, Toll-Like Receptor 4 (TLR4). Antibodies reactive with the core of LPS (EndoCAb) are produced by B cells in response to LPS stimulation, both under normal conditions and during enteric infections (11). These neutralize LPS activity and may be protective in conditions of acute endotoxemia, such as sepsis and cardiac surgery, where low basal levels of EndoCAb are associated with increased short and long-term mortality (12,13). EndoCAb levels also increase under conditions of chronic infection and inflammation, such as cystic fibrosis, where they serve as a marker for chronic LPS exposure (7,14). Each of these markers have increased half-lives compared to LPS itself (LPS = 1–3 hours, IgG EndoCAb = 10–18 hours, LBP and sCD14 = 24–48 hours) (10,11,15). Both LBP and EndoCAb are increased in adult-onset IBD patients, and have been been shown to correlate with circulating LPS (7,8). Whether this was also the case in pediatric-onset IBD was not known.

Toll-like receptors (TLR) link the innate and adaptive immune systems by recognizing conserved structures on microorganisms and through a complex cascade (involving an adaptor molecule Myd88) trigger pro-inflammatory cytokine production in efforts to maintain homeostasis; however, recent studies have shown that TLR signaling may also be required for counter-regulatory immune responses, and so in some settings could limit

inflammation (16,17). Aberrancy of the TLR signaling system may therefore regulate chronic inflammation in IBD. Moreover, our recent murine studies have shown that LPS can directly suppress growth hormone (GH) signaling, by inducing proteolytic degradation of the hepatic GH receptor (GHR) (18). LPS may therefore also contribute directly to linear growth failure in children with CD.

The role of TLR signaling has recently been investigated in both murine and human IBD. Adult and pediatric patients with UC and CD have increased TLR4 expression in colonic mucosa compared to healthy controls (19,20). Mechanistically, the role of TLR4 in this setting has not been fully characterized, although recent murine studies have suggested homeostatic effects upon epithelial regeneration and repair, and bacterial clearance (17,21). Most recently, an adoptive transfer model of colitis utilizing Myd88 deficient mice (adaptor molecule for numerous TLRs) illustrated the importance of the TLR system directly upon pro-inflammatory T cell function (22). We chose to examine effects of TLR4 signaling upon mucosal T cell responses and injury in the Trinitrobenzene-sulfonic acid (TNBS) murine model of colitis. This model utilizes a haptenizing agent that allows for both study of acute mucosal injury and activation of the acute phase response (APR), and an effector T cell response which targets the enteric flora.

In this study, we found that pediatric patients with IBD have evidence for increased systemic endotoxin response/exposure (CD greater than UC), and that in CD this is associated with linear growth failure at diagnosis and following therapy. In the murine model, we found that TLR4 was required for activation of the systemic APR following TNBS administration, as well as a relative expansion of regulatory T cells. Collectively, these studies suggest that TLR4 signaling may contribute to both systemic complications of IBD including linear growth failure, and local counter-regulatory responses.

## MATERIALS AND METHODS

### Human Subjects

Our studies included 104 patients with pediatric-onset CD (72 males; mean age, 11.3 yr [range 1–18 yr], 43 patients with pediatric-onset UC (24 males; mean age, 11.7 yr [range 2–18 yr]) and 24 pediatric controls (18 males; mean age, 11.6 yr [range 3–18 yr]). Diagnosis was based on established clinical, radiological, and endoscopic criteria, supported in all cases by histopathology. Controls were enrolled from children undergoing diagnostic evaluation for IBD and found to have normal tissue. The ultimate diagnoses in the controls were either functional abdominal pain or irritable bowel syndrome.

Clinical disease activity was determined by a retrospective chart review performed by a reviewer blinded to the serum EndoCAb and LBP results. We utilized the criteria employed by Griffiths et al in a previous report of growth and clinical course in pediatric CD (23). This defined four categories of clinical disease activity: quiescent, mild, moderate, or severe. For the cross-sectional evaluation of the relationship between clinical disease activity and serum EndoCAb and LBP, patients with mild, moderate, or severe activity at the time of the blood draw were classified as active. For the longitudinal assessment of disease activity and growth, patients were classified as moderate-to-severe if they experienced this level of activity at any point during six month blocks from diagnosis through month 48, or most recent follow-up if less than 48 months. Disease location (L) and behavior (B) for CD were determined according to the Montreal classification scheme (24). Records were reviewed and surgery related to IBD was recorded. Height (cm) and weight (kg) were converted to Z scores using CDC normative charts for age and gender. Growth retardation was defined as Z score $< -1$ for age and gender.

### Animals

*TLR4*-deficient mice (backcrossed 10 generations) and wild-type (WT) controls both on a C57/BL6J background were maintained under specific pathogen-free conditions at the animal facility of Cincinnati Children's Hospital Research Foundation. Mice were allowed free access to autoclaved feed and to drinking water. All mice used were sex-matched and between 6 and 8 weeks of age.

### Induction of TNBS Colitis

Colitis was induced by rectal installation of 2,4,6-trinitrobenzene sulfonic acid in 100 mcl 50% ethanol (ETOH) (TNBS; Sigma Chemical Co., St. Louis, MO) (25). Mice received either 125 mg/kg (high dose) or 50 mg/kg (low dose) on day zero and were sacrificed on day 3 or day 6. Controls received the same volume of 50% ETOH. Mortality and weight loss were recorded.

### Histological scoring of distal colon

Colons were excised and the distal colonic tissue was fixed with 10% neutral formalin, paraffin embedded, sectioned at 3–6 μm, and stained with hematoxylin and eosin in the UC Pathology Core. Sections were analyzed in a blinded fashion. Scoring was performed according to Neurath, et al (25).

### ELISA assays

Blood samples were collected under sterile conditions into endotoxin-free tubes, and serum was obtained and stored at −80°C until analysis. Serum lipopolysaccharide binding protein (LBP;Hycult Biotechnology, Uden**,** Netherlands), endotoxin core IgA antibody (EndoCAb IgA; Hycult Biotechnology, Uden**,** Netherlands) and soluble CD14 (sCD14; Quantikine, R&D Systems, Minneapolis, MN) were determined by solid-phase, enzyme-linked immunosorbant sandwich assay as per manufacturer's instructions. Murine blood samples were collected by cardiac puncture into endotoxin-free tubes, and serum was obtained and stored at −80°C until analysis.

### Cincinnati Cytokine Capture Assay

Biotynylated antibodies to IFNγ, IL-6, TNFα and IL-10 were injected intraperitoneally 24 hours prior to harvest as per our prior published method (26). Serum was collected at harvest and enzyme-linked immunosorbant assay was performed according to our prior published method (26).

### Flow cytometry (FACS) analysis for characterization of mesenteric lymph node (MLN) cell populations

Fresh single cell suspensions were prepared from mesenteric lymph nodes (MLN) and spleen. Cells were stained for surface markers anti-mouse CD3/CD4/CD25/CD44/CD62L (BD Biosciences, San Jose, CA and eBiosciences, San Diego, CA) and intracellular staining for anti-mouse Foxp3 (eBiosciences, San Diego, CA) following manufacturer's recommendations. Cells were acquired on a FacsCaliber BD flow cytometer and analyzed with Cell Quest BD software.

### Data Analysis

Statistical analysis was performed using GraphPad PRISM Version 4.01. Continuous variables were analyzed using two-sample t test or ANOVA with Bonferroni test for multiple comparisons, or for non-parametric alternatives, the Mann-Whitney test or Kruskal-Wallis with Dunns test for multiple comparisons. Differences in frequencies between groups

were tested using the Fisher Exact Test. A Kaplan-Meier curve with log rank analysis was performed to test for differences in mortality in the animal model. Differences in disease activity between groups over time in the patient-based study were tested by two-way ANOVA.

## ETHICAL CONSIDERATIONS

Informed consent was obtained from all families and the Cincinnati Children's Hospital Institutional Review Board approved the study protocol. The Cincinnati Children's Institutional Animal Care and Use Committee approved all animal studies.

## RESULTS

### Clinical and demographic characteristics of the IBD patients

The clinical and demographic characteristics of the IBD patients and controls are summarized in Table I. The majority of the IBD patients and controls were male. The relative frequency of males within the CD group (69%) exceeded that within the UC group (56%, p=0.05), and did not differ from the controls (75%). The mean age at diagnosis (11.3 vs. 11.7 years) and duration of disease (39.5 months vs. 35.7 months) was comparable between the CD and UC groups. The majority of the IBD patients had clinically inactive disease at the time of blood sampling, and inflammatory disease behavior with ileo-colonic involvement. Twenty-one percent had required at least one surgery.

### Serum EndoCAb IgA, LBP, and sCD14

We first asked whether circulating markers of endotoxin exposure and the APR would be elevated in IBD patients compared to controls. The mean (SEM) serum EndoCAb IgA concentration was increased from 27.19 ± 5.5 AU/mL in controls to 53.98 ± 8.1 AU/mL in UC and 146.6 ± 20 AU/mL in CD (see Fig. 1A, $p < 0.001$ for CD vs. both UC and controls). CD patients had significantly higher serum EndoCAb IgA concentration than UC patients ($p < 0.001$). The mean (SEM) serum LBP concentration was increased from 14.07 ± 1.08 μg/mL in controls to 29.66 ± 3.1 μg/mL in UC and 38.16 ± 2.6μg/mL in CD (see Fig. 1B, $p < 0.001$ for UC and CD vs. controls). The mean (SEM) serum sCD14 concentration did not differ between UC, CD and controls (2328 ± 195.7, 2124 ± 161.8, and 2619 ± 229.1 ng/mL, respectively).

### Circulating EndoCAb and LBP Relative to CD Activity, Location and Behavior

We then asked whether these markers for endotoxin exposure and the APR would be differ within the CD group based upon disease activity, location, or behavior. Both CD patients in remission (99.23 ± 19.34 log AU/mL, $p <0.001$) and those with active disease (128 ± 62.09 log AU/mL, $p<0.001$) at the time of the blood draw had significantly elevated serum EndoCAb IgA concentration compared to controls (see Fig. 1C). The mean (SEM) serum LBP concentration was also significantly increased in CD patients with active disease (45.9 ± 5.71 μg/mL) and those in clinical remission (39.26 ± 3.16 μg/mL) compared to controls, and did not differ between the two CD groups (see Fig. 1D, $p< 0.001$ for CD in remission vs. control). The serum EndoCAb IgA and LBP concentrations did not vary within the CD group with either disease location or behavior (data not shown). Consistent with this, CD patients who required surgery versus those who did not also did not vary for either mean (SEM) serum LBP concentration (34.99 ± 3.9μg/mL and 39.05 ± 3.15 μg/mL, respectively) or mean (SEM) serum EndoCAb IgA concentration (150.4 ± 36.95 AU/mL and 145.5 ± 23.59 AU/mL, respectively). Morever, these parameters did not vary with gender within the control, UC, or CD groups (data not shown). Collectively, these data confirmed that

circulating markers of endotoxin exposure and the APR were elevated in pediatric-onset CD patients, and that these persisted even while in clinical remission.

## Markers of Endotoxin Exposure and the APR and Linear Growth

We then asked whether linear growth or weight gain varied as a function of circulating EndoCAb IgA or LBP. The follow-up period was from diagnosis to study entry, and was equal to a mean (SD) of 4.1 (3.6) years. We did not observe a difference in HTz at follow-up as a function of age at diagnosis, gender, frequency of moderate-to-severe disease activity during follow-up, or exposure to infliximab therapy during follow-up (see Supplemental Figure 1.) We did not observe a linear correlation between serum EndoCAb IgA or LBP concentration and either HTz or WTz, (see Figs. 2A and 3A). However, the nonparametric correlation (Spearman) between serum LBP and HTz approached significance ($r=-0.21$, $p=0.06$). Therefore, a categorical analysis was performed to determine whether linear growth would vary between CD groups with high versus low values of serum EndoCAb or LBP. We stratified by the median serum EndoCAb IgA (88 AU/mL) or LBP (33 mcg/mL) concentration within the CD group (Lo vs Hi) and compared linear growth and weight gain. As shown in Table II, CD patients with elevated serum EndoCAb IgA were more likely to be female than those with lower serum EndoCAb IgA. Otherwise, age at diagnosis, race, disease location or behavior, surgery rates, or medication exposures did not vary by serum EndoCAb IgA or LBP concentration within the CD group. At diagnosis, 44% of the CD patients with elevated serum EndoCAb IgA exhibited linear growth retardation (defined as $HTz < -1$), compared to 23% of the group with lower serum EndoCAb IgA (see Fig. 2B, $p=0.05$). In contrast to the difference in linear growth, weight at diagnosis did not differ between the two groups (see Fig. 2B). This suggested that increased endotoxin exposure was associated with a specific effect upon linear growth, independent of overall malnutrition at diagnosis.

We then asked whether differences in the APR, as measured by serum LBP, would be associated with differences in linear growth. Height z score at diagnosis and during follow-up was reduced in CD patients with elevated serum LBP (LBP Hi), compared to those with lower serum LBP (LBP Lo) (see Fig. 3B). At diagnosis, 49% of the CD patients with elevated serum LBP exhibited linear growth retardation (defined as $HTz < -1$), compared to 26% of the group with lower serum LBP (see Fig. 3C, $p=0.01$). At follow-up, this relative difference in linear growth retardation persisted ($p=0.05$), although both groups had experienced an improvement relative to diagnosis. In contrast to the difference in linear growth, there were no differences in weight either at diagnosis or during follow-up between the two groups (see Figs. 3D and 3E). Importantly, reduced height z score persisted in the LBP Hi group despite normalization of weight z score.

Clinical disease activity was assessed over six month periods from diagnosis through month 48, or most recent follow-up. The frequency of moderate-to-severe clinical disease activity did not differ between the groups over the first 24 months following diagnosis (see Fig. 3F). However, there was an increased frequency of moderate-to-severe activity in the group with elevated LBP from month 30 through 48 ($p=0.01$ by two-way ANOVA). Collectively, these studies demonstrated that a marker of the systemic APR, LBP, is associated with linear growth retardation in children with CD, in the absence of differences in weight gain.

## Circulating LBP and Cytokine Levels following TNBS Administration to WT and TLR4 Deficient Mice

We then asked whether endotoxin signaling via TLR4 was required for systemic up-regulation of the pro-inflammatory cytokines TNFα and IL-6, and LBP, in the animal model of colitis due to TNBS administration. High dose TNBS administration led to severe colitis

with a high level of mortality and weight loss which did not differ by genotype (data not shown). Therefore, low dose TNBS was utilized for the remainder of the studies to test the effect of TLR4 signaling upon the systemic APR to minor epithelial injury and antigenic exposure in the colon. The mean (SEM) circulating LBP concentration was increased to 84 ± 4 mcg/mL in WT mice at day three following TNBS administration, compared to 43 + 10 mcg/mL in ETOH-treated controls ($p < 0.01$, see Fig. 4A). By day six, the serum LBP concentration had returned to basal levels in WT mice. While circulating LBP trended towards an increase following TNBS administration in *TLR4* deficient mice at day 3, this did not reach significance, compared to TLR4 deficient controls, and was significantly lower than WT mice at day 3 ($p<0.05$). Both serum TNFα (797.5 ± 266.1 pg/mL) and IL-6 (21703 ± 7185 pg/mL) were significantly increased at day three in WT mice compared to ETOH-treated controls (196.7 ± 53.9 pg/mL and 1602 ± 445.9 pg/mL, respectively, $p<0.05$ and $<0.01$, see Figs. 4B and 4C). Neither cytokine was significantly increased at day three in *TLR4* deficient mice, and both were significantly lower than WT controls at this time point ($p<0.01$). Weight loss following TNBS administration has been linked to activation of systemic immunity and central anorexia leading to reduced chow intake. Both WT and *TLR4* deficient mice experienced maximal weight loss by day 3, and had recovered weight by day 6 (see Fig. 4D). However, maximal weight loss was substantially lower in the *TLR4* deficient mice compared to the WT controls, consistent with the reduction in serum LBP, TNFα, and IL-6 production.

### Serum IL-10 and IFNγ Production and Severity of Colitis following TNBS Administration

Recent studies have linked TLR4 signaling to both counter-regulatory and pro-inflammatory T cell responses. We found that the mean (SEM) serum IL-10 concentration increased significantly in WT mice (898 ± 119.7 pg/mL) and, though not significant, also increased in *TLR4* deficient mice (873 ± 248.6 pg/mL) at day three, compared to 219.5 ± 61.45 pg/mL and 183.4 ± 87.1 pg/mL, respectively, in ETOH-treated controls (see Fig. 5A). The increase in serum IL-10 persisted at day six in WT mice, but returned to basal levels at this time point in *TLR4* deficient mice. The converse was observed for IFNγ, which increased from 137 ± 59 pg/mL in ETOH-treated controls to 16018 ± 8964 pg/mL at day three in *TLR4* deficient mice (see Fig. 5B, $p < 0.01$), and exhibited only a modest increase, to 3298 ± 1694 pg/mL, in WT mice. We then asked whether these differences in circulating IL-10 and IFNγ would be associated with a difference in mortality or colon histology. We found that mortality did not vary between the WT and *TLR4* deficient mice (data not shown). Neither the WT nor *TLR4* deficient mice exhibited a significant increase in histological injury three days after low dose TNBS administration, compared to controls which received ETOH alone (see Fig. 5C). However, by day six after TNBS administration, the mean (SEM) histological score was significantly increased in the *TLR4* deficient mice, to 1.8 ± 0.3**,** compared to 0.66 ± 0.15 at day three and 0.78 ± 0.08 in controls who received ETOH alone (see Fig. 5C, $p < 0.01$ and $<0.05$ respectively). The histological abnormality in the *TLR4* deficient mice at day six included crypt hyperplasia, mucin depletion, and a mononuclear cell infiltrate.

### T-Lymphocyte Responses in WT and *TLR4 −/−* mice following TNBS Administration

We then asked whether these differences in circulating IL-10 and IFNγ would be associated with a difference in the frequency of regulatory and activated/memory T cells in the draining mesenteric lymph nodes (MLN) or spleen. Three and six days after low dose TNBS administration, we observed a modest increase in the frequency of $CD4^+CD44^+CD62L^{lo}$ activated/memory T cells in the MLN of both WT and *TLR4* deficient mice which did not reach statistical significance (see Fig. 6A). By day six, the frequency of splenic $CD4^+CD44^+CD62L^{lo}$ activated/memory T cells had increased in both groups. By comparison, the frequency of $CD4^+FOXP3^+$ regulatory T cells was significantly increased in the MLN of both groups at days three and six ($p < 0.001$ and $p < 0.01$, respectively; see

Fig. 6B). This included an increase in both the $CD25^+$ and $CD25^-$ populations. However, there was a greater relative increase (approximately two-fold higher) in this population in the WT mice compared to the *TLR4* deficient mice at day three ($p < 0.01$). Therefore, the overall MLN Treg/Teff ratio was significantly higher in the WT mice compared to the *TLR4* deficient mice at this timepoint ($p < 0.05$; see Fig. 6C). The splenic Treg response did not differ between the groups. Taken together, these data suggested that TLR4 signaling was required for a counter-regulatory response to a mild TNBS-induced antigenic exposure in the gut, which involved IL-10 production and expansion of $FOXP3^+$ T cells in the MLN. When this response was reduced by *TLR4* deficiency, a mild colitis ensued.

## DISCUSSION

Recent studies in adult-onset IBD have linked susceptibility in part to genotypic variation in *TLR4*, and have described increased circulating levels of endotoxin and markers of endotoxin exposure in patients with active disease (7,8,27). Whether this would also be the case in pediatric-onset IBD was not known. We found that pediatric patients with IBD exhibit increased systemic endotoxin responses which are greater for those with CD compared to UC. Surprisingly, these endotoxin responses persisted despite clinical remission, and were independent of disease location or behavior. Recent studies in patients with HIV infection have characterized a defect in gut permeability in patients with progressive disease, leading to increased bacterial translocation and chronic immune activation (28). Considering a similar barrier defect occurs in CD, it is conceivable that similar effects on immune activation exist. Taken together, our data suggest that endotoxin may therefore be a key contributor to both chronic inflammation and extra-intestinal complications including growth failure in pediatric IBD.

Linear growth failure is common at diagnosis of CD, and often persists despite normalization of nutritional status. This has suggested the presence of an inflammatory growth hormone (GH) resistance. We have recently determined that endotoxin will induce GH resistance in mice via proteolytic degradation of the GH receptor (GHR), and have previously reported that TNFα neutralization will rapidly restore GH signaling in murine colitis, prior to resolution of mucosal inflammation (18,29). We found that linear growth retardation was more common in CD patients with evidence of endotoxin exposure, as suggested by elevated serum EndoCAb IgA, and an increased APR, as demonstrated by elevated serum LBP. Importantly, weight z scores did not differ between these groups, suggesting that linear growth failure was not simply due to malnutrition. Whether endotoxin directly induces GH resistance via GHR proteolysis in CD patients is not known, and is the subject of our ongoing studies. However, it would appear likely that the subset of CD patients with persistent endotoxin exposure and a chronically activated APR are most likely to experience permanent growth failure, and could benefit from therapies targeting this pathway. Persistent elevation of serum LBP may therefore serve as a useful biomarker for identifying pediatric CD patients with the highest likelihood of experiencing refractory growth failure. As LBP is induced by both LPS and inflammatory cytokines such as IL-6, it will be important in future studies to confirm that these are in fact chronically increased in children with CD and growth failure.

As endotoxin signals through TLR4, we sought to characterize its role in the initiation of the systemic APR utilizing an experimental colitis model which encompasses both innate and adaptive features. The TNBS model was selected because of the T-cell dependency of the ensuing colitis, and because the role of TLR4 signaling in this model has not previously been explored. In addition, this model has been shown by gene array to mimic human disease (30). We found that markers of endotoxin exposure and the APR persisted at high levels in pediatric CD patients despite clinical remission. We therefore chose to utilize a low

dose of TNBS based upon prior studies by our group and others, and carefully accounted for the weight of the mice (31,32). By doing so, we sought to test the impact of intact TLR signaling upon the systemic APR and weight loss following minor colonic epithelial injury and antigenic exposure.

As a means of confirming the utility of this model in investigating the endotoxin dependent APR and for comparison with our patient-based studies, LBP was measured. To our knowledge, this has not previously been measured in a murine model of colitis. With striking similarity to the IBD patients, WT mice exhibited elevated circulating levels of LBP three days after low dose TNBS administration. LBP returned to basal levels by day 6. We then utilized a novel technique to test for TLR4 dependent differences in the associated APR. The Cincinnati capture assay allows quantification of integrated cytokine production over a 24 hour period. Biotinylated cytokine-specific antibodies are administered to mice 24 hours prior to sacrifice, and “capture” all of the cytokine which is released into the serum over that time period (26). Our data support a critical role for TLR4 in the APR to TNBS administration. We have identified for the first time a TLR4-dependent increase in both LBP and the pro-inflammatory cytokines TNFα and IL-6 at day three, which returned to basal levels by day six. This was associated with a substantial reduction in weight loss in *TLR4* deficient mice compared to WT controls. Collectively, these studies have confirmed that intact TLR4 signaling is required for systemic induction of pro-inflammatory cytokines and LBP expression, and associated weight loss, following minor colonic injury. This raises the intriguing possibility that the reduction in linear growth observed in association with increased markers of endotoxin exposure and the APR in children with CD is also dependent upon intact TLR4 signaling.

Conversely, we found that the Th1 cytokine IFNγ was induced in *TLR4*-deficient mice following TNBS administration, relative to a more modest up-regulation in WT controls. We therefore asked whether there might also be a TLR4 dependent difference in the magnitude or kinetics of counter-regulatory IL-10 production. We found that TNBS administration led to a robust increase in IL-10 production at day three in both WT and *TLR4* deficient mice. This persisted at day six in WT mice, but returned to basal levels in *TLR4* deficient mice, coincident with the development of mild colitis. These data are consistent with recent studies in transfer models which have demonstrated that TLR signaling via myd88 may regulate T cell responses in colitis (22).

Considering the differences found in IFNγ and IL-10 production, we then focused upon phenotyping the MLN T cells. A modest increase in the frequency of MLN activated/memory T cells which did not reach significance was noted in both *TLR4* deficient and WT mice. A more significant increase in the frequency of MLN FOXPP3+ regulatory T cells was noted in WT mice, relative to a blunted response in *TLR4* deficient mice. When considering the ratio of MLN regulatory to activated/memory T cells, this was significantly increased in WT mice, and abrogated in *TLR4* deficient mice. Moreover, as the activated/memory and regulatory T cell response to TNBS in the spleen did not vary by *TLR4* genotype, these differences appeared to be specific for local MLN responses. This would be consistent with studies showing that TLR4 is required for the development of tolerance to food antigens in the gut (33), and that minor non-specific epithelial injury in the colon can trigger an antigen-specific regulatory T cell response (16). Future studies will define the functional properties of the foxp3+ T cells isolated from WT and *TLR4* deficient mice.

Taken together, our results support a role for endotoxin and intact TLR4 signaling in both pediatric and murine colitis. Our patient-based studies have for the first time identified an association between endotoxin exposure, activation of the APR, and linear growth failure which persists during therapy of pediatric CD. Our data in the animal model have confirmed

that intact TLR4 signaling is required for robust up-regulation of TNFα, IL-6, and LBP following minor colonic injury. However, our experimental data also suggest that TLR4 may exert an acute protective effect in the gut related to its role in IL-10 production and counter-regulatory T cell responses. These divergent effects of TLR4 signaling will need to be accounted for as therapies are developed to more specifically target the endotoxin:TLR4 pathway in IBD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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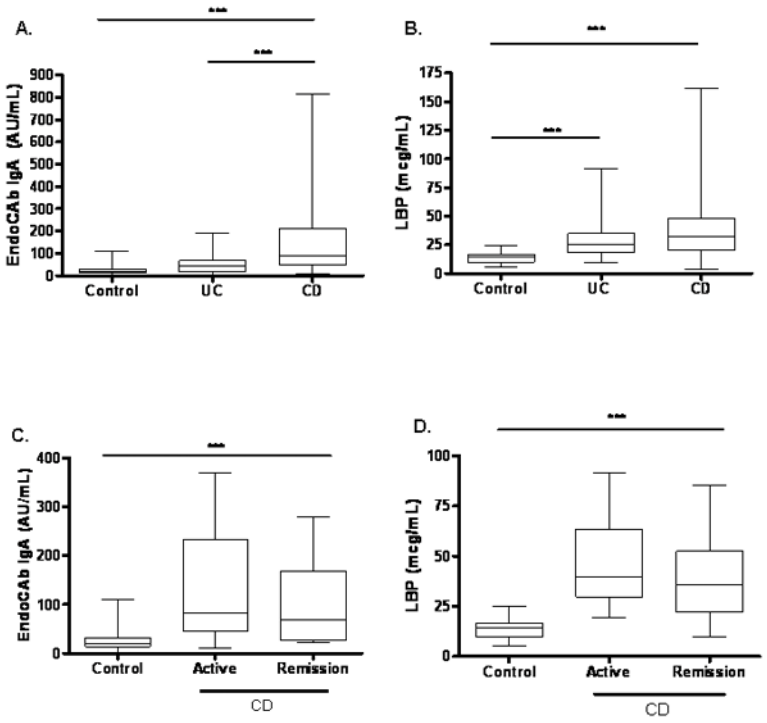



**Figure 1. Serum EndoCAb IgA and LBP Concentration in Pediatric IBD**

(A) Serum EndoCAb IgA concentration was determined in CD (n=65) and UC (n=34) patients and healthy controls (n=20) by ELISA. ***p < 0.001 by Kruskal-Wallis with Dunn's multiple comparison test. (B) Serum LBP concentration was determined in CD (n=95) and UC (n=32) patients and healthy controls (n=21) by ELISA. ***p < 0.001 by Kruskal-Wallis with Dunn's multiple comparison test. (C) Serum EndoCAb IgA concentration was determined in CD patients stratified by disease activity at the time of blood sampling (n=23) and healthy controls (n=20) by ELISA. ***p < 0.001 by unpaired t test. (D) Serum LBP concentration was determined in CD patients stratified by disease activity at the time of blood sampling (n=51) and healthy controls (n=21) by ELISA. ***p < 0.001 by unpaired t test. Data are shown as median (interquartile range) and overall range.

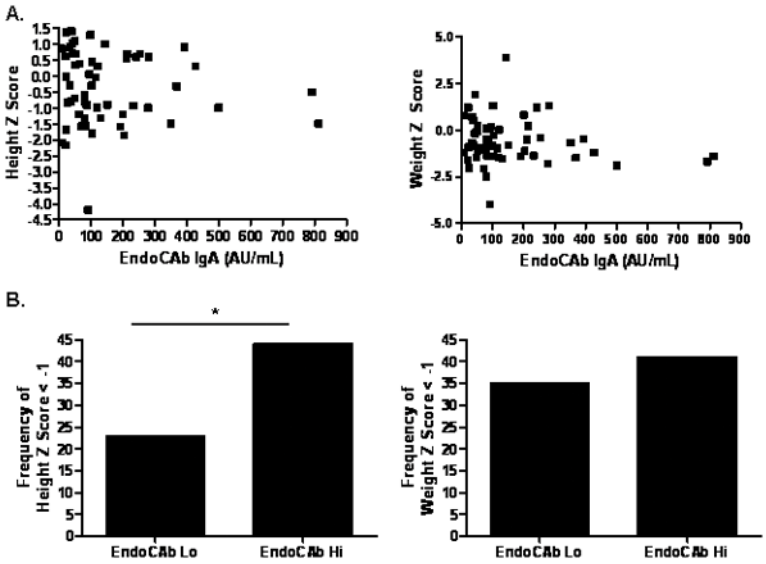



**Figure 2. Serum EndoCAb IgA Concentration and Linear Growth at Diagnosis**

A) HTz and WTz at diagnosis within the CD group are shown relative to serum EndoCAb IgA. B) CD patients were stratified by the median serum EndoCAb IgA concentration (88 AU/mL) (n=54, Lo vs Hi), and the frequency of growth retardation at diagnosis defined as z score for age and gender < −1 was determined. Data are shown as the frequency of growth retardation for height or weight. *p≡ 0.05 by Fisher exact test.

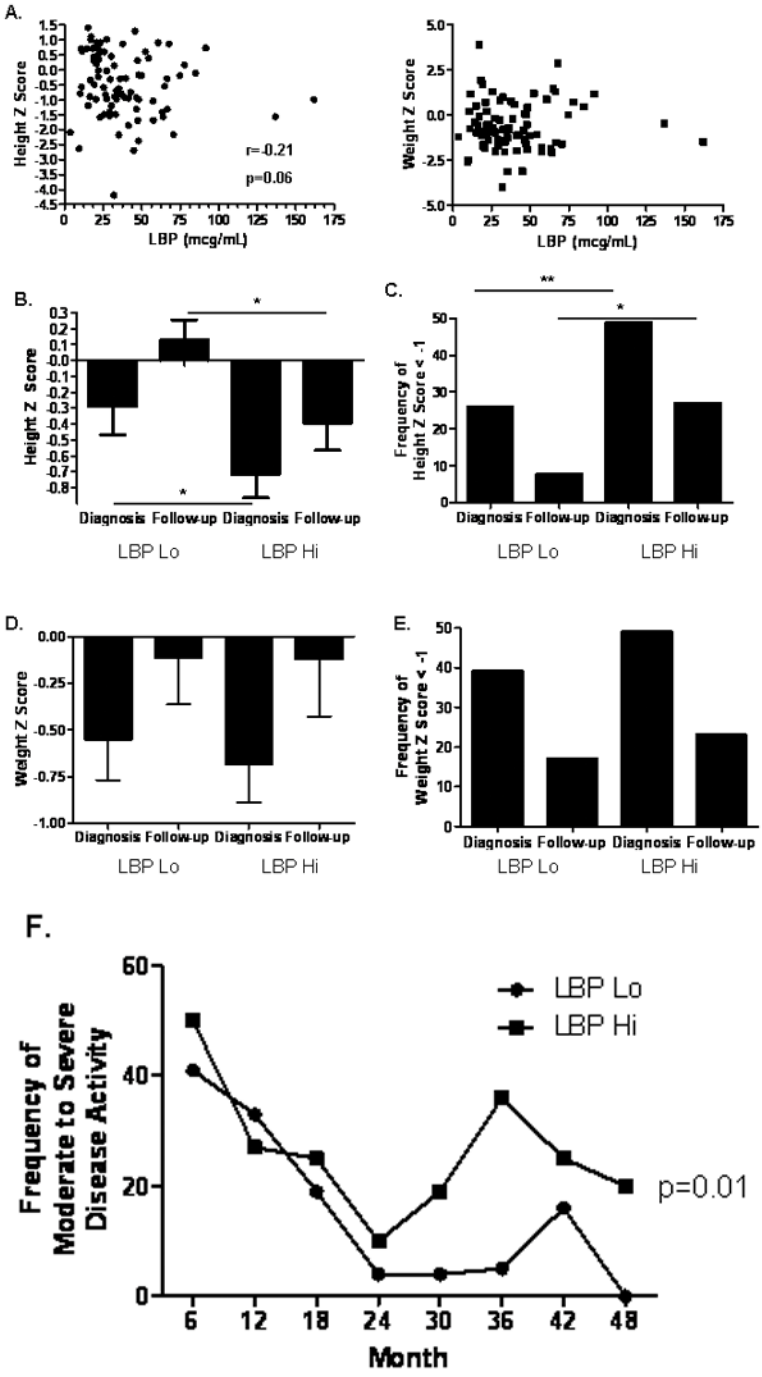



**Figure 3. Serum LBP Concentration and Linear Growth**

A) HTz and WTz within the CD group are shown relative to serum LBP. CD patients were stratified by the median serum LBP concentration (33 mcg/mL, Lo vs Hi), and height and weight z scores and the frequency of growth retardation defined as z score for age and gender < −1 were determined at diagnosis and most recent follow-up (n=83 and 74, respectively). Data are shown as the mean (SEM) for (B) height z score, *p < 0.05 by Mann-Whitney test, or (D) weight z score, or the frequency of growth retardation for (C) height, **p= 0.01 by Fisher exact test, *p=0.05 by Fisher exact test, or (E) weight. (F) The

frequency of moderate-to-severe clinical disease activity was determined over six month intervals from diagnosis through month 48 and is shown, p=0.01 by two-way ANOVA.

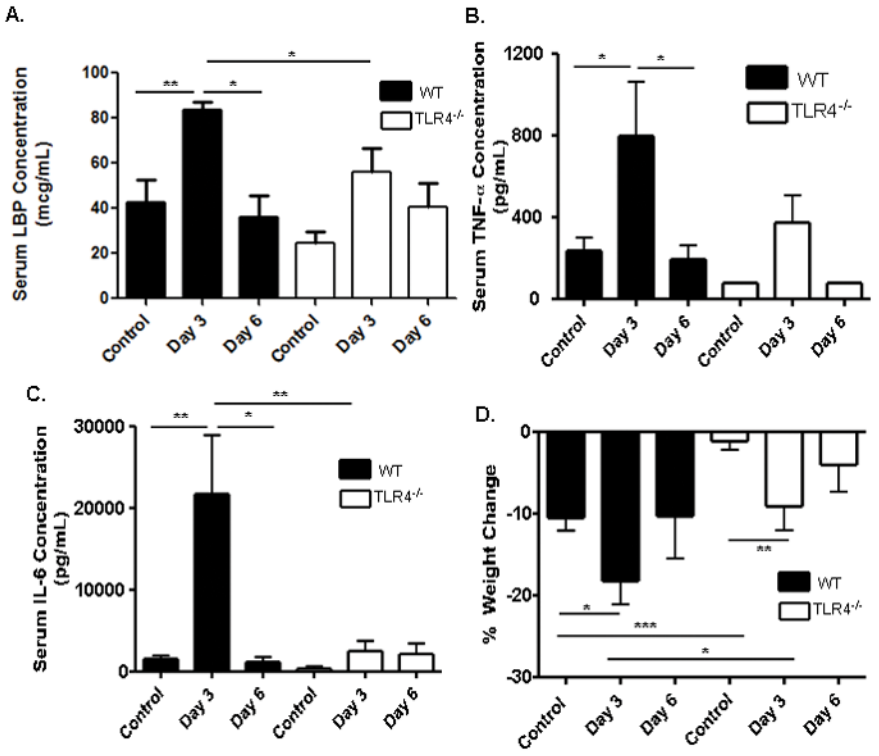



**Figure 4. Circulating LBP, Pro-Inflammatory Cytokines, and Weight Loss Following TNBS Administration to WT and TLR4 deficient mice**

(A) Serum LBP concentration was measured at day 3 in WT (n=4) and TLR4 deficient (TLR4−/−) (n=6) mice, as well as WT (n=6) and TLR4−/− (n=3) control mice and at day 6 in WT (n=4) and TLR4−/− (n=3) mice by ELISA. (B) TNF-α was measured at day 3 in WT (n=4) and TLR4−/− (n=5) mice, as well as WT (n=13) and TLR4−/− (n=4) control mice and at day 6 in WT (n=4) and TLR4−/− (n=6) mice by ELISA. (C) IL-6 was measured at day 3 in WT (n=4) and TLR4−/− (n=7) mice, as well as WT (n=13) and TLR4−/− (n=4) control mice and at day 6 in WT (n=4) and TLR4−/− (n=6) mice by ELISA. (D) Weight change from baseline was recorded for WT (n=11) and TLR4 −/− (n=11) mice. Data are shown as the mean (SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by unpaired t test or Mann-Whitney test.

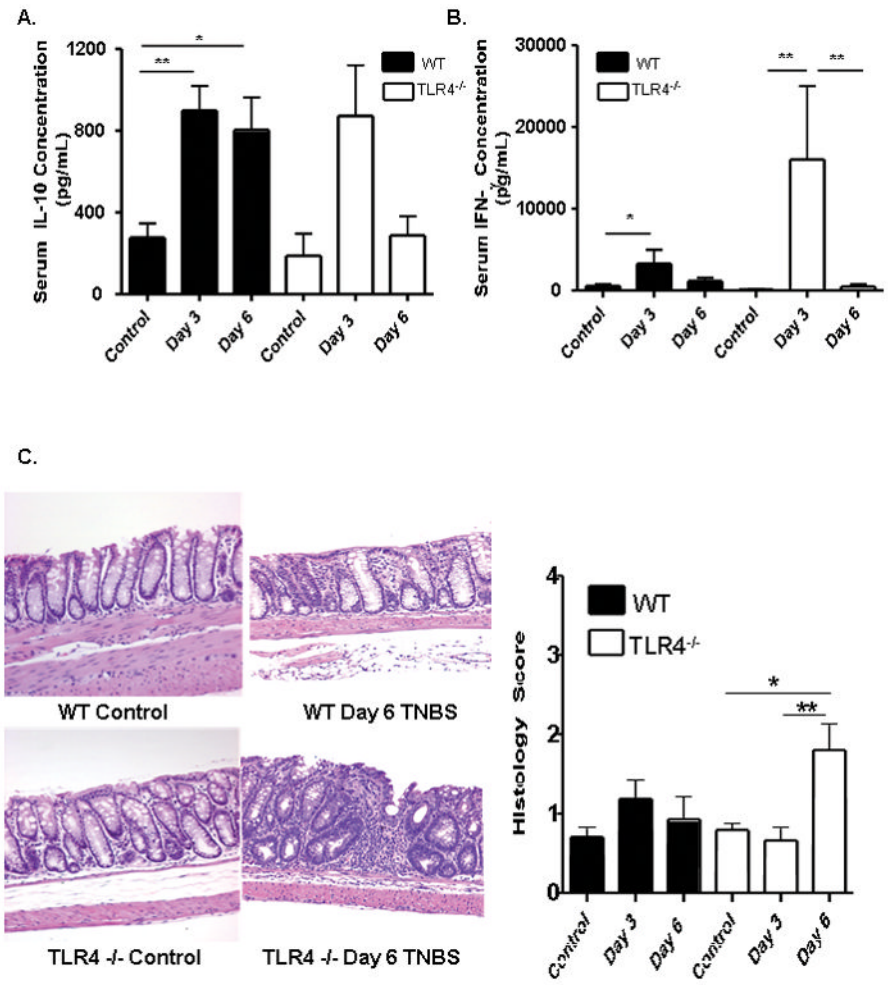
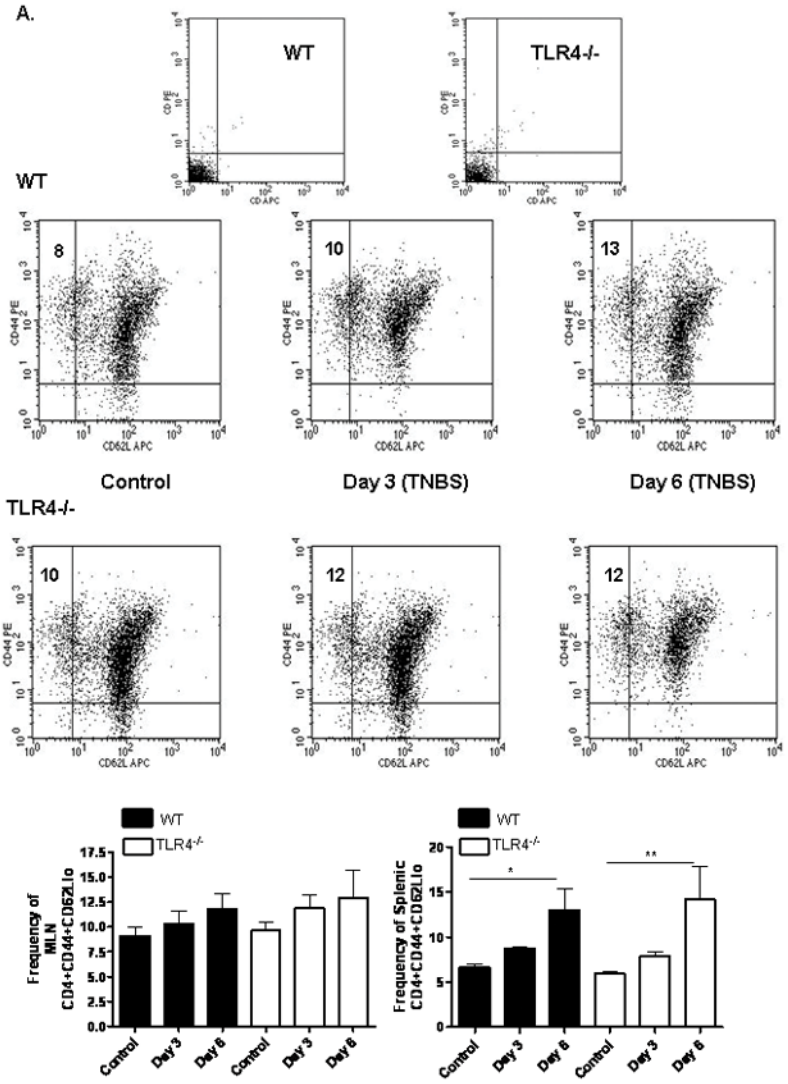



**Figure 5. Circulating IL-10 and IFNγ and Colon Inflammation Following TNBS Administration to WT and TLR4 −/− mice**

TNBS (50mg/kg) was administered to WT and TLR4 deficient mice at day 0. (A) IL-10 was measured at day 3 in WT (n=4) and TLR4−/− (n=6) mice, as well as WT (n=13) and TLR4−/− (n=4) control mice and at day 6 in WT (n=4) and TLR4−/− (n=6) mice by ELISA. (B) IFN-γ was measured at day 3 in WT (n=4) and TLR4−/− (n=6) mice, as well as WT (n=13) and TLR4−/− (n=4) control mice and at day 6 in WT (n=4) and TLR4−/− (n=6) mice by ELISA. (C) Representative distal colon sections from WT (n= 5) and TLR4 −/− (n=6) mice at day 6 are shown. Histologic scores of WT (n=6) and TLR4−/− (n=9) mice harvested at day 3 and WT (n= 5) and TLR4 −/− (n=6) mice harvested at day 6 as well as WT (n=20) and TLR4 −/− (n=23) controls were recorded.*p <0 .05, **p < 0.01, by unpaired t-test. Data are shown as the mean (SEM).



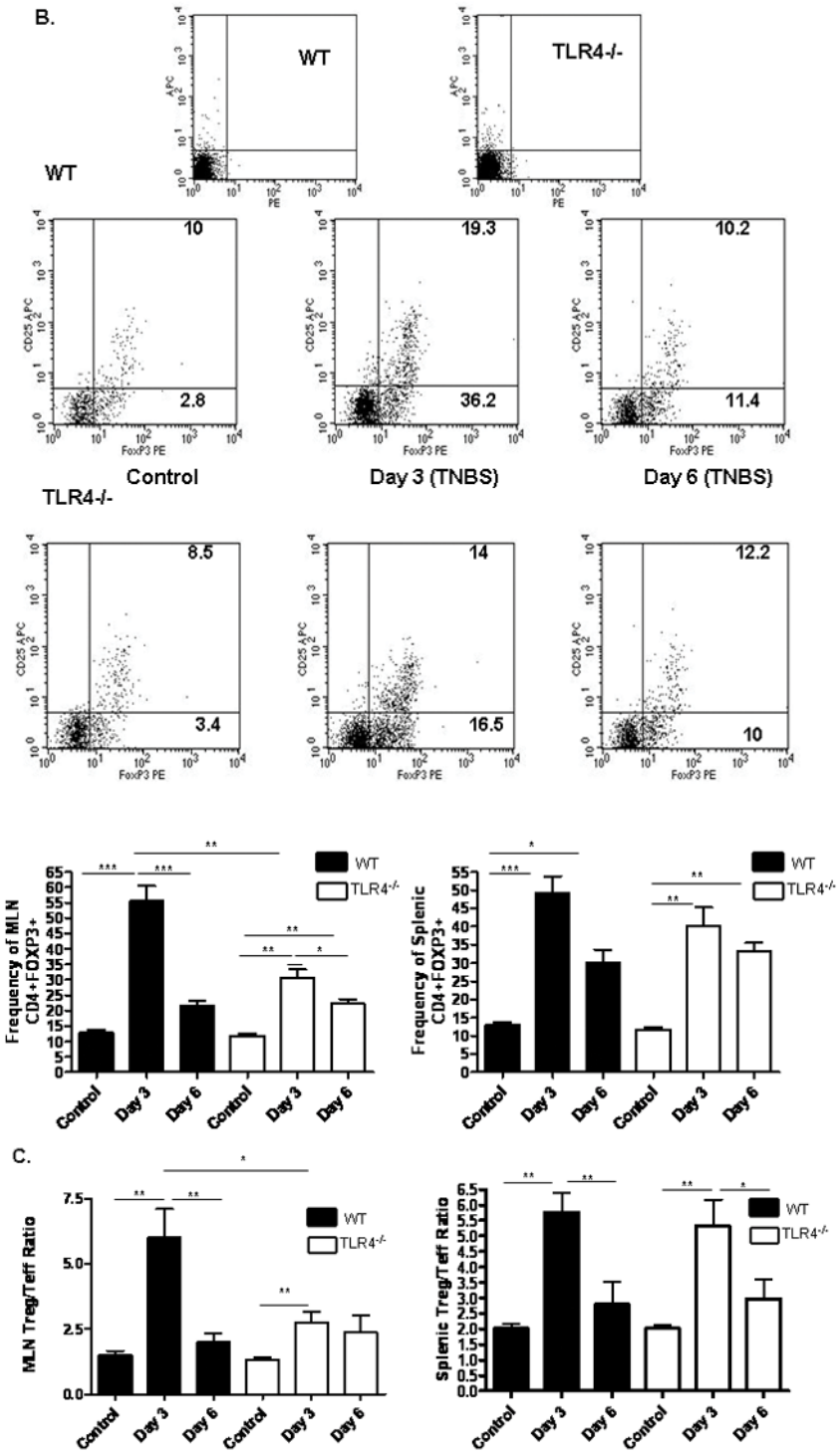



**Figure 6. TLR4 Dependent T Lymphocyte Responses to TNBS Administration**

(A) The effect of TNBS administration upon the frequency of mesenteric lymph node (MLN) or splenic $CD44^{+}CD62L^{lo}$ activated/memory T lymphocytes was determined by flow cytometry at days 3 and 6 from WT (n=6) and TLR4 −/− (n=6) mice, as well as WT (n=8) and TLR4 −/− (n=10) controls. Representative scatter graphs from MLN for each group are shown, as well as the mean (SEM). The first two scatter graphs are for the isotype

controls used to set the gates. (B) The frequency of MLN or splenic $CD4^{+}foxp3^{+}$ regulatory T lymphocytes was determined by flow cytometry at days 3 and 6 from WT (n=5) and TLR4 −/− (n=6) mice, as well as WT (n=8) and TLR4 −/− (n=10) controls. Representative scatter graphs from MLN for each group are shown, as well as the mean (SEM). The first two scatter graphs are for the isotype controls used to set the gates. (C) The Treg/Teff ratio was calculated for each mouse from the MLN or splenic $CD4^{+}foxp3^{+}$ regulatory T lymphocyte and $CD44^{+}CD62L^{-}$ activated/memory T lymphocyte frequencies. The mean (SEM) for each group was as shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by ANOVA with Bonferroni's multiple correction test.

## Table I

Clinical and Demographic Characteristics.

| | Crohn's Disease | Ulcerative Colitis | Control |
|---|---|---|---|
| # of patients | 104 | 43 | 24 |
| Mean age (range) [years] | 11.3 (1–18) | 11.7 (2–18) | 11.6 (3–18) |
| Gender (male) | 69% | 56%* | 75% |
| Mean duration (range) [months] | 39.5 (1–176) | 35.7 (1–160) | - |
| **Disease activity** | | | - |
| Active (%) | 26% | 28% | - |
| **Disease location** | | | - |
| | L1: 11% | - | - |
| | L2: 17% | - | - |
| | L3: 72% | - | - |
| **Disease behavior** | | | - |
| B1 (%) | 69% | - | - |
| B2 or B3 (%) | 31% | - | - |
| Surgery (%) | 21% | - | - |

*p=0.05 vs. CD

L1:Ileal location

L2:Colon-only location

L3:Ileo-colonic location

B1:Non-stricturing, non-penetrating behavior

B2:Stricturing behavior

B3:Internal penetrating behavior

## Table II

Clinical and Demographic Characteristics Stratified by Serum EndoCAb and LBP Concentration.

| | EndoCAb Lo | EndoCAb Hi | LBP Lo | LBP Hi |
|---|---|---|---|---|
| Mean age (range) | 11(3–18) | 12(5–17) | 12(3–17) | 12(5–18) |
| Gender (male) | 81% | 53%* | 63% | 71% |
| Race (white) | 97% | 84% | 91% | 96% |
| **Disease location** | | | | |
| | L1: 19% | L1: 9% | L1: 15% | L1: 11% |
| | L2: 10% | L2: 19% | L2: 20% | L2: 15% |
| | L3: 71% | L3: 72% | L3: 65% | L3: 74% |
| **Disease behavior** | | | | |
| B1 (%) | 74% | 62% | 78% | 70% |
| B2 or B3 (%) | 26% | 38% | 22% | 30% |
| Surgery (%) | 26% | 28% | 22% | 22% |
| **Medications** | | | | |
| Prednisone | 83% | 84% | 76% | 89% |
| 6-Mercaptopurine/ | 83% | 90% | 85% | 77% |
| Azathioprine | | | | |
| Infliximab | 48% | 44% | 35% | 43% |

*p=0.01 vs. EndoCAb Lo

L1:Ileal location

L2:Colon-only location

L3:Ileo-colonic location

B1:Non-stricturing, non-penetrating behavior

B2:Stricturing behavior

B3:Internal penetrating behavior