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Early administration of probiotic *Lactobacillus acidophilus* and/or prebiotic inulin attenuates pathogen-mediated intestinal inflammation and Smad 7 cell signaling

Ondulla T. Foye[1], **I-Fei Huang**[1,2,3], **Christine C. Chiou**[2,3], **W. Allan Walker**[1], and **Hai Ning Shi**[1]

[1]Mucosal Immunology Laboratory, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

[2]Department of Pediatrics, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan

[3]National Yang-Ming University, Taipei, Taiwan

Abstract

Immaturity of gut-associated immunity may contribute to pediatric mortality associated with enteric infections. A murine model to parallel infantile enteric disease was used to determine the effects of probiotic, *Lactobacillus acidophilus* (La), prebiotic, inulin, or both (synbiotic, syn) on pathogen-induced inflammatory responses, NF-κB, and Smad 7 signaling. Newborn mice were inoculated bi-weekly for 4 weeks with La, inulin, or syn and challenged with *Citrobacter rodentium* (Cr) at 5 weeks. Mouse intestinal epithelial cells (CMT93) were exposed to Cr to determine temporal alterations in NF-Kappa B and Smad 7 levels. Mice with pretreatment of La, inulin, and syn show reduced intestinal inflammation following Cr infection compared with controls, which is associated with significantly reduced bacterial colonization in La, inulin, and syn animals. Our results further show that host defense against Cr infection correlated with enhanced colonic IL-10 and transforming growth factor-β expression and inhibition of NF-κB in syn-treated mice, whereas mice pretreated with syn, La, or inulin had attenuation of Cr-induced Smad 7 expression. There was a temporal Smad 7 and NF-κB intracellular accumulation post-Cr infection and post-tumor necrosis factor stimulation in CMT93 cells. These results, therefore, suggest that probiotic, La, prebiotic inulin, or synbiotic may promote host-protective immunity and attenuate Cr-induced intestinal inflammation through mechanisms affecting NF-κB and Smad 7 signaling.

Keywords

probiotics; prebiotics; synbiotic; enteric pathogens; cell signaling

Correspondence: Hai Ning Shi, Mucosal Immunology Laboratory, Massachusetts General Hospital, Building 114, 16th Street, Room 3504, Charlestown, MA 02129, USA. Tel.: +1 617 726 4173; fax: +1 617 726. 4172; shiha@helix.mgh.harvard.edu.
Present address: Ondulla T. Foye, FDA, Center for Food Safety and Applied Nutrition, 8301 Muirkirk Rd, MOD-1, Office 2007, Laurel, MD, 20708, USA

Authors' contribution: O.T.F. and I.-F.H. contributed equally to this work.

Introduction

In the last two decades, diarrheal illnesses have accounted for approximately 4.6 million deaths of 1 billion episodes of diarrhea globally in children younger than 5 years (Snyder & Merson, 1982; Institute for World Health, 2010, http://www.oneworldhealth.org/diarrheal_disease). Pediatric mortality and morbidity remain a constant epidemiological problem owing to immaturity of gut-associated immunity (GAI) and subsequent resistance to enteric pathogens. These conditions predominate during early childhood and do not appear during any other stage of life (Snyder & Merson, 1982; Hoque *et al.*, 1994), highlighting the particular vulnerability of the intestine during early development. Infections caused by enteric bacterial pathogens, such as diarrheagenic enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli*, the family of attaching and effacing (A/E) bacterial pathogens, are among the most important causative pathogens of severe infantile diarrhea (Donnenberg & Whittam, 2001; Hecht, 2001; Vallance *et al.*, 2002). The mouse pathogen *Citrobacter rodentium* causes a similar A/E lesion in the murine intestine and has been used as a physiological model of human infection of EPEC and EHEC *E. coli*. Using the *C. rodentium* model, we have shown that preinoculation of murine gut with *Lactobacillus acidophilus*, a probiotic strain, early in life can enhance host defense against enteric bacterial infection and attenuate bacteria-mediated intestinal injury (Chen *et al.*, 2005). We also observed that probiotic treatment stimulates regulatory cytokine expression in the colon transforming growth factor (TGF-β) (Chen *et al.*, 2005). In line with these observations, it has been shown that breast-fed infants have a greater resistance to enteric pathogens owing to the transfer of commensal bacteria (Fanaro *et al.*, 2003), nondigestible oligosaccharides (Newburg *et al.*, 2005), TGF-β in maternal milk (Saito *et al.*, 1993), and immunoglobulins (Brandtzaeg, 2010) which enhance development ofthe GAI. Moreover, targeted colonization of the neonate intestine with commensal microbiota has been shown to be effective in allergy prevention in later infancy (Lodinová-Zádníková *et al.*, 2010). More specifically, the intestinal microbial communities predominately induce the maturation of the mucosal adaptive immune system in the human neonate (Kaplan *et al.*, 2011). Conversely, formula-fed infants lack maternal transfer of commensal bacteria, nondigestive oligosaccharides, and TGF-β which results in the modification of gut microbial communities compounding the vulnerability of the neonatal intestine to enteric pathogens (Le Huërou-Luron *et al.*, 2010).

TGF-β is a very potent negative regulator of mucosal inflammation (Letterio & Roberts, 1998) inhibiting T cell activation (Letterio, 2005) vital to maintaining tolerance to innocuous antigens found within the intestine. TGF-β mediates cell signaling by ligand-dependent activation of heterodimeric transmembrane serine/threonine kinases receptors (Piek *et al.*, 1999). Downstream, the ligand-activated receptor directly phosphorylates Smad2 and Smad3 proteins, which associate with Smad 4 and translocate to the nucleus to participate in transcriptional control of targeted genes (Heldin *et al.*, 1997). Disruption of TGF-β signaling occurs in the presence of antagonistic Smad 7, an inhibitor for TGFb signaling, which physically interferes with activation of Smad 2/Smad3 by preventing the interaction with TGF-β receptor (Hayashi *et al.*, 1997) leaving epithelial cells of the intestine in a state of

enhanced expression and production of pro-inflammatory cytokines (Maggio-Price *et al.*, 2006).

Excessive Smad 7 protein blocks TGF-β signaling and maintains elevated pro-inflammatory cytokines in inflammatory bowel disease (IBD) patients, while silencing Smad7 expression restores the anti-inflammatory effects of TGF-β (Monteleone *et al.*, 2001; Nguyen & Snapper, 2009). Additionally, IBD patients have high nuclear factor Kappa B (NF-κB) (Jobin and Sartor, 2000) and Smad7 protein expression (Monteleone *et al.*, 2001, 2004a, b, c; Nguyen & Snapper, 2009), which may be correlated with enhanced chronic colonic inflammation. Several studies have suggested a strong correlation between NF-κB and TGF-β/Smad pathways (Bitzer *et al.*, 2000; Nagarajan *et al.*, 2000; Haller *et al.*, 2003). In lamina propria mononuclear cells isolated from IBD patients, abrogation of Smad7 with antisense oligonucleotides allowed endogenous TGF-β to up-regulate inhibitor Kappa B-alpha (IκB-α) and lower NF-κB accumulation (Monteleone *et al.*, 2004c).

The probiotic (commensal intestinal microorganisms)-induced effect on the NF-κB signaling pathway is well established (Yoon and Sun, 2011). Sougioultzis *et al.* (2006) reported that *Saccharomyces boulardii*, nonpathogenic yeast, inhibited interleukin 8 (IL-8) production, IκB-α degradation, reduced NF-κB DNA binding, and NF-κB reporter gene up-regulation of interleukin 1 (IL-1) in intestinal cells *in vitro*. Oral administration of probiotics attenuate intestinal inflammation (Petrof *et al.*, 2004; Tien *et al.*, 2006; Mañé *et al.*, 2009) and NF-κB activation induced by infection (Murphy *et al.*, 2008), stress, tumor necrosis factor (TNF-α), and interleukin 1 (Petrof *et al.*, 2004).

Previously, we reported that inoculation of the probiotic *L. acidophilus* enhanced enteric protection to pathogens and reduced mucosal inflammation by enhancing TGF-β expression in mice (Chen *et al.*, 2005). In the current study, by utilizing both *in vivo* (*C. rodentium*-mouse model, a model of human infection of EPEC and EHEC *E. coli*) and *in vitro* approaches, we tested the hypothesis that early inoculation of probiotic *L. acidophilus* may enhance host-protective immunity to enteric bacterial pathogens through promoting TGF-β response, which exerts its anti-inflammatory effect by reducing Smad 7 expression, allowing TGF-β to up-regulate IκB-α and lower NF-κB accumulation, and that co-administration of prebiotics, the nondigestible food ingredients, which can stimulate the growth and/or activity of beneficial probiotic bacteria, may promote probiotic-induced anti-inflammatory effects.

Materials and methods

Mice

Six- to 8-week-old female and male BALB/c ByJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME), bred in a specific pathogen-free facility at Massachusetts General Hospital (Charlestown, MA), and provided mouse chow and sterile water *ad libitum*. Neonatal mice were born to pregnant female Balb/c ByJ mice. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Bacterial cultures

Probiotic *L. acidophilus* (La) was cultured in deMan, Rogosa, and Sharpe broth (MRS; Difco, Detroit, MI) and grown at 37 °C for 20 h and re-suspended in PBS prior to oral inoculation (1×10^9 CFU per mouse). *Citrobacter rodentium* (strain DBS100; American Type Culture Collection number 51459) was grown overnight in Luria broth (LB) and subsequently re-suspended in PBS prior to dosing (0.5 mL per mouse; approximately 5×10^8 CFU of *C. rodentium* per mouse). *Citrobacter rodentium* (Cr) antigen was prepared by collecting an overnight culture of Cr in LB. The bacterial culture was washed in PBS and sonicated on ice. The homogenate was then centrifuged (6000 ***g***) at 4 °C for 30 min. Supernatants were collected, and the protein concentration was determined.

In vivo experimental design

Three independent experiments were conducted in which neonatal (3 days of age) mice and lactating dams were randomly divided into five groups of approximately 7–10 pups per treatment (Fig. 1): group A (nontreated normal control mice), group B (*C. rodentium* inoculated), group C (prebiotic inulin treated + *C. rodentium*), group D (probiotic *L. acidophilus* + *C. rodentium*), group E (synbiotic combination probiotic *L. acidophilus* + prebiotic inulin + *C. rodentium*). Mice of treatment group D were administered *L. acidophilus* (approximately 1×10^9 CFU per mouse) twice weekly by intragastric gavage for approximately 7 weeks. Sterile water was supplemented with prebiotic: inulin and oligofructose (1 g per 100 mL, Raftilose Synergy®) and administered by intragastric gavage three times weekly from 1 to 3 weeks of age and administered in drinking water provided *ad libitum* from weeks 3 to 7 weeks of age for mice of treatment group C, with fresh inulin-supplemented drinking water provided every 2 days. Mice of treatment group E were administered a synbiotic combination of *L. acidophilus*, approximately 1×10^9 CFU per mouse and prebiotic inulin (1 g per 100 mL) by intragastric gavage two times per week from 1 to 7 weeks of age. Control mice (group A) only received a saline vehicle bi-weekly over the duration of the experiment. At 5 weeks of age, mice of treatment groups B, C, D, and E were orally inoculated by intragastric gavage with enteric pathogen, *C. rodentium*. All mice were sacrificed at 7 weeks of age.

Quantitation of clearance of *C. rodentium*

To assess the clearance of Cr, fecal pellets were collected from each mouse weekly postinfection. Fecal pellets were weighed, homogenized, serially diluted, and plated on selective MacConkey agar plates for gram-negative organisms (Chen *et al.*, 2005; Johnson-Henry *et al.*, 2005; Wu *et al.*, 2008). Bacterial colonies were enumerated after overnight incubation at 37 °C. *Citrobacter rodentium* colonies were easily distinguished by appearance, using MacConkey agar–plated bacterial cultures from the manufacturer (strain DBS100; American Type Culture Collection number 51459) as a positive control. Bacterial counts are reported as colony-forming units per gram.

Lymphocyte isolation

Mice were sacrificed 2 weeks post-Cr infection. Lymphocyte suspensions were prepared from the mesenteric lymph nodes (MLN) and spleen as described previously (Shi *et al.*,

2000; Chen *et al.*, 2005). Cells (5×10^6 cells mL^{-1}) were cultured on 48-well plates in the presence or absence of Cr antigen (50 μg mL^{-1}) or plate-bound anti-CD3 MAb (10 μg mL^{-1}). Culture supernatants were collected after 72 h and stored at −20 °C until assayed for cytokine production.

Measurement of interferon gamma, IL-10, and TNF-α production by ELISA

ELISA capture antibodies [R4-6A2, interferon gamma (IFN-γ); JESS-2A5, IL-10] and biotinylated secondary antibodies (XMG1.2, IFN-γ; SXC-1, IL-10) were purchased from PharMingen (San Diego, CA), whereas TNF-α ELISA capture antibodies (MP6-XT22) and biotinylated secondary antibodies (C1150-14) were purchased from BD Pharmingen, San Jose, CA. The biotinylated secondary antibodies were used as a second layer, and reactions were visualized with O-phenylenediamine at 492 nm (OPD; Zymed Labs, South San Francisco, CA). Standard curves were obtained using recombinant murine IFN-γ (Genzyme, Cambridge, MA), IL-10 (R&D Systems, Minneapolis, MN), and TNF-α (BD Pharmingen). Optical density values were converted to pg mL^{-1} for each cytokine by linear regression with Delta Soft II (Biometallics, Princeton, NJ).

Histopathological examinations

At necropsy, colonic tissues were isolated and small fragments were then frozen in Tissue-Tek® O.C.T. Compound (Miles Inc. Elkhart, IN) and stored at −80 °C. Some colonic fragments were snap-frozen in liquid nitrogen and then stored at −80 °C for detection of colonic cytokine gene expression. Seven-micrometer sections were cut on a 2800 Frigocut cryostat (Reichert-Jung, Germany) and stained with hematoxylin and eosin. Sections were analyzed without prior knowledge of treatment. Colonic pathology was scored using a modified histology scoring system based on previously published methods (Chen *et al.*, 2005). The scoring system consists of two parts. Part 1 is the determination of the infiltration of inflammatory cells in the colon, with scores ranging from 0 to 4 (0, normal cell pattern; 1, scattered inflammatory cells in the lamina propria; 2, increased numbers of inflammatory cells in the lamina propria; 3, confluence of inflammatory cells extending into the submucosa; and 4, transmural extension of the infiltrative inflammatory cells). Part 2 is the evaluation of colon tissue damage, with scores that also range from 0 to 4 (0, normal tissue pattern; 1, minimal inflammation and colonic crypt hyperplasia; 2, mild colonic crypt hyperplasia with or without focal invasion of epithelium; 3, obvious colonic crypt hyperplasia, invasion of epithelium, and goblet cell depletion; and 4, extensive mucosal damage and extension through deeper structures of the bowel wall). The total colon pathology score equals the inflammatory cell score plus the tissue damage score (Fig. 3g).

Cell culture

Mouse intestinal epithelial cell line CMT93 was grown in six-well plates with complete DMEM [10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 100 U penicillin mL^{-1}, 100 μg streptomycin mL^{-1}, 50 μM 2-mercaptoethanol, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Life Technologies, Grand Island, NY)]. All cultures were maintained at 37 °C in a humidity-controlled incubator with 5% CO_2 and were grown to confluence over 5–6 days before addition of pathogenic bacteria *C. rodentium*. The cells

were washed and placed in antibiotic-free medium for 1 h. Confluent stock monolayers were subcultured by trypsinization.

In vitro experimental design

In this study, we utilized mouse intestinal epithelial cell line CMT93 to better elucidate cell signaling responses to enteric pathogens *in vitro*. Nine experiments were conducted independently with similar results. To determine the time-dependent intracellular changes of NF-κB and Smad 7 in response to pathogen exposure, CMT93 cells were exposed with Cr (2.5×10^7 CFU per well) for 1 h in antibiotic-free DMEM at 37 °C. Subsequently, the media and cell lysates were collected at 0, 15, 30, 60, 90, and 120 min and 14 and 24 h postpathogen exposure. Cells were washed and lysed [(1% Triton-X-100 supplemented with 0.1 μM phenylmethylsulphonyl fluoride, 0.1 μM sodium orthovanadate, and Halt protease inhibitor (10 μL mL^{-1}, Pierce cat# 78410, Thermo Scientific, Rockford, IL)]. The lysates were kept at −80 °C for future Western blot analysis. The culture supernatants were stored at −20 °C for future measurement of TNF-α cytokine production.

Detection of colonic cytokine expression (quantitative real-time PCR)

Total RNA was isolated from frozen colonic tissue (distal part of the colon) and treated CMT93 cells using TRIzol (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's protocol. First-strand cDNA was synthesized using 2 μg of extracted total RNA (Ready-to-Go kit; Amersham Pharmacia Biotech, Piscataway, NJ). IL-10 and TGF-β colonic expression was determined by realtime PCR using QuantiTect SYBR green real-time PCR kit (Qiagen, Valencia, CA) on the Opticon II DNA thermocylcer (MJ Research, Waltham, MA). A PCR master mix was prepared according to the manufacturer's protocol with a reaction volume of 50 μL, using the following real-time cycler conditions: 95 °C for 15 min, 94 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s for 38 cycles. GAPDH was used as internal controls. LightCycler relative quantification software was used to normalize data to the same GAPDH mRNA level. Samples were run in duplicate. Mouse IL-10 and TGF-β commercially available PCR primers were purchased from Biosource International, Inc. (Camarillo, CA) for detection, while GAPDH commercially available upstream and downstream PCR primers were utilized for detection (R&D Systems, Minneapolis, MN).

Protein extraction and Western blot analysis

Mouse colonic tissue and treated CMT93 cells were homogenized with lysis buffer prepared as previously mentioned. The suspensions were centrifuged at 4 °C, and the supernatant was collected, and protein content was determined using DC protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by SDS-PAGE for Western blot analysis. Pooled samples per treatment [equal protein amounts (μg) from each mouse within a treatment] from colonic tissue were separated by SDS-PAGE for Western blot analysis, while lysates of 2-well replicates of treated CMT93 cells were pooled per treatment and separated by SDS-PAGE for Western blot analysis. Smad7 and IκB-α protein expression was determined using polyclonal rabbit anti-mouse Smad7 (sc-11392) and IκB-α (sc-847) primary antibodies, respectively (Santa Cruz Biotech, Santa Cruz, CA). Bio-detection was determined utilizing secondary antibody goat anti-rabbit IgG conjugated with horseradish

peroxidase (sc-2004, Santa Cruz). Each blot was stripped and analyzed for GAPDH protein expression, as an internal loading control, using a specific rabbit anti-mouse GAPDH antibody (sc25778, Santa Cruz), followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase.

Statistical analysis

All results were expressed as the mean ± SEM. Statistical differences were determined using one-way analysis of variance test (Tukey's multiple comparison test) with GRAPHPAD PRISM. A value for $P < 0.05$ was considered significant.

Results

Probiotic La and/or prebiotic inulin administration reduces *C. rodentium*-induced early morbidity in mice pups

Numerous reports have demonstrated the various health benefits of probiotic administration in mature animals (Tien *et al.*, 2006; Damaskos & Kolios, 2008; Farnworth, 2008; Gill & Prasad, 2008). However, few studies have examined the effects of administration of probiotics and/or prebiotics on early development, survivability, and resistance to enteric pathogens in young animals. To determine how early inoculation of probiotic, La, and/or prebiotic inulin may alter the developmental patterns of the GAI affecting host resistance to enteric pathogens, we pre-inoculated the mice with and without La, inulin, and both and infected them with *C. rodentium*. During the experimental period, the clinical symptoms, change in body weight and survival of the animals were monitored. As expected, mice infected only with Cr showed signs of *Citrobacter*-associated disease, such as soft stool, a hunched posture, disturbed body hair, and a marked body weight loss during the initial period of infection. The body weight remained significantly lower in mice with Cr infection alone throughout the experiment period compared with groups that were uninfected normal control ($P < 0.01$), *C. rodentium*-infected with pretreatment of probiotic La ($P < 0.05$), and synbiotic combination ($P < 0.05$) (Fig. 2a). Pretreatment of mice with prebiotic inulin alone showed limited effect on host body weight gain during *C. rodentium* infection, as the body weight changes of these mice did not differ significantly with all other treatment groups ($P > 0.05$ for all comparisons: Inu + Cr vs. Cr; Inu + Cr vs. La + Cr; Inu + Cr vs. Synb + Cr; and Inu + Cr vs. control).

Moreover, a 10% mortality rate was detected in the group that was infected with Cr alone, and no mortality was observed in any other groups (data not shown). These results provide evidence to suggest a protective effect of pretreatment of neonatal mice with probiotic La and synbiotic treatment (La + inulin) on host response against enteric bacterial infection.

To further determine effects of pretreatment of La, inulin, or both on host protection, we examined whether these treatments affected bacterial output from *C. rodentium*-infected mice by collecting the fecal pellets during the experimental periods, homogenizing, and plating them onto the commonly used selective MacConkey agar plates for the determination of the number of *C. rodentium* (Chen *et al.*, 2005; Johnson-Henry *et al.*, 2005; Wu *et al.*, 2008). Our results show that bacterial output was significantly lower in mice

pretreated with probiotic La ($P < 0.05$), prebiotic inulin ($P < 0.05$), or with both (synbiotic) ($P < 0.01$) at both 1 week postinfection (Fig. 2b). The same trend was consistent through 2 weeks postinfection (Fig. 2c) in all treatment groups with the difference in bacterial output being more pronounced in synbiotic and La group ($P < 0.001$) and prebiotic inulin treatment ($P < 0.01$). These results provide evidence indicating that the probiotic, prebiotic, and symbiotic treatments alter the dynamics of the enteric bacterial infection.

Microscopic examination showed that mice infected with *C. rodentium* showed typical pathological changes associated with this bacterial infection in the intestine, including colonic epithelial cell hyperplasia, crypt elongation, extensive inflammatory cellular infiltration, and disruption of the epithelial surface (Fig. 3a and d). Colonic tissue of mice pretreated with either probiotic La (Fig. 3b) or prebiotic inulin (Fig. 3c) showed less severe pathology (Fig. 3g) compared with mice infected with Cr alone (Fig. 3a and d). This is evidenced by milder colonic crypt elongation, less cellular infiltration of the colonic lamina propria, and epithelial damage detected in La- or inulin-treated mice (Fig. 3b and c) in comparison with Cr-infected mice (Fig. 3a and d). The pathology scores for inflammation and intestinal damage were significantly lower in probiotic La-, prebiotic inulin- and La plus inulin-treated mice, as compared to mice only infected with *C. rodentium* (Fig. 3g). These observations suggest that pretreatment of probiotic La or prebiotic inulin resulted in a reduction in bacteria-induced intestinal damage. No significant differences were detected in colonic pathology score between La- and inulin-treated mice (Fig. 3g). Furthermore, pathological analysis of colonic tissue revealed that mice pretreated with synbiotics had the most significant reduction in intestinal inflammation and intestinal damage (Fig. 3e and g), as evidenced by the mildest degree of colonic inflammation post-Cr infection in comparison with all the other treatments, with the exception of the controls (Fig. 3f). These results demonstrate that pretreatment of neonatal mice with probiotic La and/or prebiotic inulin may attenuate Cr-induced colon injury with synbiotic treatment being more effective.

Pretreatment with probiotic, La, and/or prebiotic inulin alters cytokine responses in the MLN and intestine of mice

Colonization of *C. rodentium* on the intestinal epithelial surface resulted in a Th1-type immune response, and Th1 cytokines play a role in host-protective immunity (Simmons *et al.*, 2002); Chen *et al.*, 2005; Gonçalves *et al.*, 2001). To test the hypothesis that early inoculation of probiotic La and/or prebiotic inulin may alter developmental patterns of the GAI, Th1, Th2, and T reg cytokine production and expression in the intestine- and gutassociated lymphoid tissue in young mice following pathogen challenge were determined. Analysis of bacterial (Cr) antigen (Cr-Ag)-specific cytokine production of the MLN revealed that the lymphocytes from mice pretreated with probiotic La, prebiotic inulin, or the synbiotic combination of probiotic La and prebiotic inulin had significantly enhanced Cr-Ag-specific IL-10 secretion (Fig. 4a) compared with that detected in mice with *C. rodentium* infection alone. Pretreatment of mice with the synbiotic combination of probiotic La and prebiotic inulin resulted in a more pronounced IL-10 production by the MLN cells compared with other groups (Fig. 4a). In contrast, the MLN of mice pretreated with the synbiotic combination of probiotic La and prebiotic inulin had significantly reduced Cr-Ag-specific IFN-γ response (Fig. 4b) at 2 weeks post-Cr infection.

To further determine the impact of La, inulin, and combined treatments on pro-inflammatory and regulatory cytokine responses in the colonic tissue, we measured gene expression of IL-10 and TGF-β, the regulatory cytokines, using real-time PCR. The results showed that mice of the synbiotic combination treated group had significantly greater colonic expression of TGF-β, in comparison with *C. rodentium*-infected control, prebiotic- and probiotictreated groups (Fig. 5a), and pretreatment of mice with La only resulted in an increase in colonic TGF-β expression. These observations, therefore, suggest that probiotic La and synbiotics enhance the expression and production of TGF-β, a key regulator of immunity and vital for the suppression of enteric pathogen-induced inflammatory responses. Similarly, probiotic La and synbiotic combination treatments resulted in a significant increase in colonic IL-10 expression (Fig. 5b) in comparison with Cr infected alone.

Citrobacter rodentium activate NF-κB and Smad 7 intracellular signaling in colonic epithelial cells *in vitro*

TGF-β can act as a potent negative regulator of mucosal inflammation. However, Smad 7, by physically interfering with activation of Smad2/Smad 3 and preventing their interaction with TGF-β, causes disruption of TGF-β signaling. This may contribute to the enhanced pro-inflammatory responses in the intestine (Hayashi *et al*, 1997; Maggio-Price *et al.*, 2006). Studies have suggested that NF-κB (Jobin & Sartor, 2000) and Smad 7 (Monteleone *et al.*, 2001, 2004b) are up-regulated in IBD patients and may be responsible for colonic inflammation. NF-κB plays a key role in regulating the immune response to infection and inflammation. In unstimulated cells, NF-κB is complexed with inhibitor, IκB-α, thereby retaining NF-κB within the cytoplasm. Upon induction of the NF-κB pathway by inflammatory signals (IL-1, TNF-α, lipopolysaccharides, stress), IκB-α is degraded; leaving NF-κB free to translocate to the nucleus to elicit transcriptional response (Gosh, 2007). Thus, we next determined the kinetics of NF-κB by measuring IκB-α protein abundance at different time points after *C. rodentium* exposure using CMT93 cells. NF-κB activation was observed at 60 min post-*C. rodentium* infection, as indicated by IκB-α degradation (Fig. 6a) in CMT93 cells. This response occurs between 30–60 min postpathogen exposure, with IκB-α levels returning to baseline within 120 min in CMT93 cells. Western blot analysis of the effects of *C. rodentium* infection on Smad 7 signaling showed a gradual increase in intracellular Smad 7 (between 0–24 h postinfection) in mouse epithelial cells (Fig. 6b), providing evidence to suggest that enteric bacterial infections induce Smad 7 expression in intestinal epithelial cells. Our analysis of TNF-α production reveals that Cr bacteria-induced NF-κB activation and Smad 7 response correlate with pro-inflammatory cytokine responses in intestinal epithelial cells. As shown in Fig. 6b, TNF-α production was enhanced at 1 h postinfection and peaked at 1.5 h post-Cr infection in CMT93 cells (Fig. 6b).

We next determined whether pro-inflammatory cytokine secretion downstream of NF-Kappa B signaling may be responsible for the induction of Smad 7 and other inflammatory signaling responses. To test this idea, CMT93 cells were stimulated with TNF-α at doses 0.63–10.0 ng mL^{-1} for 3 h and Smad 7 levels were examined using immunoblot. As indicated in Fig. 6c, a modest increase in the levels of Smad 7 was detected in most of TNF-α-treated cells (1.25, 2.5 and 5 ng mL^{-1}) in comparison with the baseline levels detected in control cells. The effect of TNF-α treatment was found to be more pronounced in cells

treated with high doses of TNF-α ng mL^{-1} CMT93 cells. These results, therefore, suggest a role of pro-inflammatory cytokines in the induction of Smad 7 expression.

Probiotic (La) and/or prebiotic (inulin) inhibit induction of the NF-Kappa B pathway and Smad 7 signaling *in vivo*

Our data from *in vitro* experiments suggest that enteric pathogen, *C. rodentium* induced intracellular NF-κB and Smad 7 signaling in intestinal epithelial cells (Fig. 6). Therefore, in our next set of studies we determine whether probiotic La, prebiotic inulin, or synbiotic pre-treatment will alter pathogen-induced NF-κB and Smad 7 signaling *in vivo*. We pretreated mice with probiotic La, prebiotic inulin, or both and infected the mice with *C. rodentium* at 5 weeks of age. Mouse colonic tissues from each group of mice were collected for immunoblotting. Our Western blot analysis revealed that mice infected with Cr alone or in combination with probiotic La or prebiotic inulin pretreatment had decreased levels of IκB-α in comparison with uninfected mice, which indicates an activation of the NF-κB pathway; suggesting that pretreatment with the probiotic La, or prebiotic inulin alone had no clear effect on attenuating NF-κB activation. By contrast, synbiotic treatment restored IκB-α to levels similar to those observed in uninfected animals (Fig. 7). The results further imply that Cr infection induces Smad 7 expression, which is inhibited in mice with pretreatment of probiotic La, prebiotic inulin, or both (Fig. 7). These results suggest that synbiotic combination of probiotic La and prebiotic inulin treatment result in the inhibition of bacteria-induced NF-κB activation and up-regulation of Smad 7 *in vivo*.

Discussion

During the early neonatal period, the human infant has a deficiency in antigen presenting cell functions (Tonon *et al.*, 2002; Darmochwal-Kolarz *et al.*, 2004; Upham *et al.*, 2009) and altered T cell-mediated immune responses (Liu *et al.*, 2001; Darmochwal-Kolarz *et al.*, 2004). However, it is during the early neonatal period that the intestine is colonized with approximately 100 trillion bacteria (Ogra & Welliver, 2008). Early exposure to environmental microorganisms promotes the maturation and development of the infant's gut and GAI and may determine the outcome to induced mucosal inflammation (Sjögren *et al.*, 2009), resistance to enteric pathogens, disease development (Hoque *et al.*, 1994), autoimmunity and allergic disorders (Isolauri & Salminen, 2008; Rodriguez *et al.*, 2010) in later life. The diversity of acquired neonatal microbiota is dependent upon the external environment microbial communities, breastfeeding (Kaplan *et al.*, 2011), use of antibiotics, and the presence of nondigestible sugars (prebiotics) in the maternal milk (Newburg *et al.*, 2005; Newburg, 2009). Upon transit to the lower gut, nondigestible oligosaccharides (prebiotics) alter the intestinal luminal environment favorable to support the growth and proliferation of commensal microorganisms. Hence, early exposure to commensal organisms (probiotics) in the breast-fed neonate enhances development and maturation of the gut and GAI and resistance to enteric pathogens (Chen *et al.*, 2005; Salminen & Isolauri, 2008). However, the precise mechanisms by which the microbial communities influence the maturation of the mucosal immunity are not fully understood. In this current study, we utilized the murine *C. rodentium* model, a physiological model of human infection of EPEC and EHEC *E. coli*, to determine how early inoculation of probiotic La and/or prebiotic

(inulin) affects intestinal innate and adaptive immunity and cell signaling molecules postpathogen exposure.

In this study, neonatal (3 days) mice pups were orally dosed with probiotic bacteria La and/or prebiotic inulin and then exposed to enteric bacterial pathogen *C. rodentium* to parallel a period of critical early development of GAI and subsequent enteric pathogen exposure in the human neonate. Our results provide evidence that protection activated by synbiotic combination of probiotic La and prebiotic inulin in *Citrobacter*-infected mice is associated with enhanced mucosal immune responses evidenced by an increase in mucosal IL-10 secretion, up-regulation of IL-10 and TGF-β mRNA expression, reduction in pro-inflammatory cytokine IFN-γ secretion, and reduced bacterial loads, which parallels findings of Steed *et al.* (2010) demonstrating a significant reduction in intestinal pro-inflammatory TNF-α expression in synbiotic-treated patients. Moreover, the results from this investigation provide evidence to suggest that early treatment with synbiotic combination of probiotic La and prebiotic inulin can effectively prevent pathogen-induced intestinal inflammation by affecting NF-κB and Smad 7 signaling within the intestinal epithelium.

Prebiotics are known to help colonization of beneficial probiotics. While early administration of a synbiotic combination of probiotic La and prebiotic inulin attenuated the secretion and expression of pro-inflammatory cytokines and inflammation, supporting a potential indirect role of prebiotic inulin in regulating mucosal immune response by modulating the colonic microbial communities. Our results are supported by previous observations showing that a diet supplemented with Fructooligosaccharides (FOS) and inulin can trigger and stimulate the gut mucosal immune system (Benyacoub *et al.*, 2008). Our observations also are in line with the results of randomized controlled trials, which provide evidence to suggest that synbiotic therapy can be more effective in the treatment IBD than therapies limited to probiotics or prebiotics (Fujimori *et al.*, 2009; Macfarlane *et al.*, 2009; Steed *et al.*, 2010). In the current study, we found that prebiotic (inulin) treatment of young mice resulted in a reduction in fecal *C. rodentium* output after the bacterial infection (Fig. 2b and c). It was reported previously that feeding rats with an inulin-oligofructose diet resulted in reduced numbers of *Salmonella* Typhimurium in the content of ileum and cecum (Kleessen & Blaut, 2005). However, contradicting results have also been reported. Petersen *et al.* (2009) reported that BALB/c mice fed diets containing prebiotics (FOS or xylo-oligosaccharide) had significantly higher numbers of *S.* Typhimurium, translocated into liver, spleen, and MLN compared with mice fed with control diet. In contrast, no increased translocation of *S.* Typhimurium was found in mice fed inulin (Petersen *et al.*, 2009), in that same study. Nevertheless, most prebiotics and/or probiotics have not been shown to cause illness, but additional research is needed to determine the safety of prebiotics and probiotics in young children or people whose immune system is compromised.

The observations showing an enhanced colonic TGF-β and IL-10 responses in mice with early synbiotic or probiotic treatments provided evidence to support the idea that these treatments may modulate gut mucosal inflammatory responses by promoting immunological regulatory mechanisms, which parallel results by Roller *et al.* (2004) demonstrating that

synbiotic and prebiotic supplementation stimulated IL-10 production in the gut-associated lymphoid tissues of azoxymethane treated rats. The protective role of IL-10 and TGF-β/Smad cascade is supported by a study showing that colonization with gram-positive *Enterococcus faecalis* in IL-10-deficient mice resulted in the development of persistent activation of TLR/NF-κB signaling and inflammation in intestinal epithelial cells, which completely lack Smad 7 expression (Ruiz *et al.*, 2005).

Smad 7 can cause disruption of TGF-β signaling by physically interfering with activation of Smad2/Smad 3 and preventing their interaction with TGF-β receptor. In the current study, we observed that mice infected with *C. rodentium* alone had significantly enhanced Smad 7 expression and pro-inflammatory cytokine secretion. These responses were reduced in mice pretreated with probiotic La, prebiotic inulin, and synbiotic combination. The association between the attenuation of pathogeninduced colitis and abolished pro-inflammatory Smad 7 signaling in colonic tissues of Cr pathogen-infected mice provide evidence to suggest that probiotic La, prebiotic inulin, and a synbiotic combination may enhance host protection from enteric pathogens by modulating regulatory immunological responses within the gut, which is supported by recent evidence demonstrating a direct effect of Smad 7 on NF-κB (Grau *et al.*, 2006). Hegazy & El-Bedewy (2010) demonstrated that oral probiotic supplementation ameliorated colonic pro-inflammatory cytokine secretion and TNF-α and NF-κB expression in IBD patients. Moreover, we demonstrate that in vitro with CMT93 cells that Smad 7 and NF-κB induction parallels pro-inflammatory cytokine secretion (TNF-α), which imply that colonic Smad 7 and NF-κB induction may be correlated with the production of inflammatory cytokines contributing to the pathological changes attributed to pathogen invasion. Other studies have also shown a correlation between chronic inflammation, pro-inflammatory cytokines, and Smad 7 in patients with autoimmune disease (Monteleone *et al.*, 2004a; Hegazy & El-Bedewy, 2010). Thus, we can conjecture that pro-inflammatory cytokines produced *in vivo* by the early responding antigen presenting cells may perpetuate Smad 7 signaling culminating in a chronic inflammatory response.

Studies have demonstrated that lamina propria mononuclear cells isolated from IBD patients had enhanced Smad 7 protein levels and pro-inflammatory cytokine secretion, which was not reduced by TGF-β, whereas inhibition of Smad 7 restores the ability of TGF-β to inhibit pro-inflammatory cytokine production (Monteleone *et al.*, 2001), implying that the effects of TGF-β in the microenvironment are not linearly related to its relative abundance. Inhibitory Smads, such as Smad 7, control the strength of the signal from the cell surface to the nucleus and thus control cell function (Monteleone *et al.*, 2001). These studies raise the exciting possibility that resolution of chronic inflammation in the gut might be accomplished by enabling endogenous immunosuppressive mechanisms to function, rather than blocking proinflammatory pathways directly. Reducing Smad 7 enables the abundant TGF-β in inflamed tissues to become functional. Consequently, in this study, we demonstrate that synbiotics not only enhanced TGF-β expression, but also reduced Smad 7 protein levels in colonic tissue of Crinfected mice, resulting in an attenuated mucosal inflammatory and immune responses. Thus, this study may help additionally to identify Smad 7 as a key pro-inflammatory cell signaling molecule altered by probiotic La, prebiotic inulin, and synbiotic administration in the presence of enteric pathogens and gut-associated inflammation.

Acknowledgments

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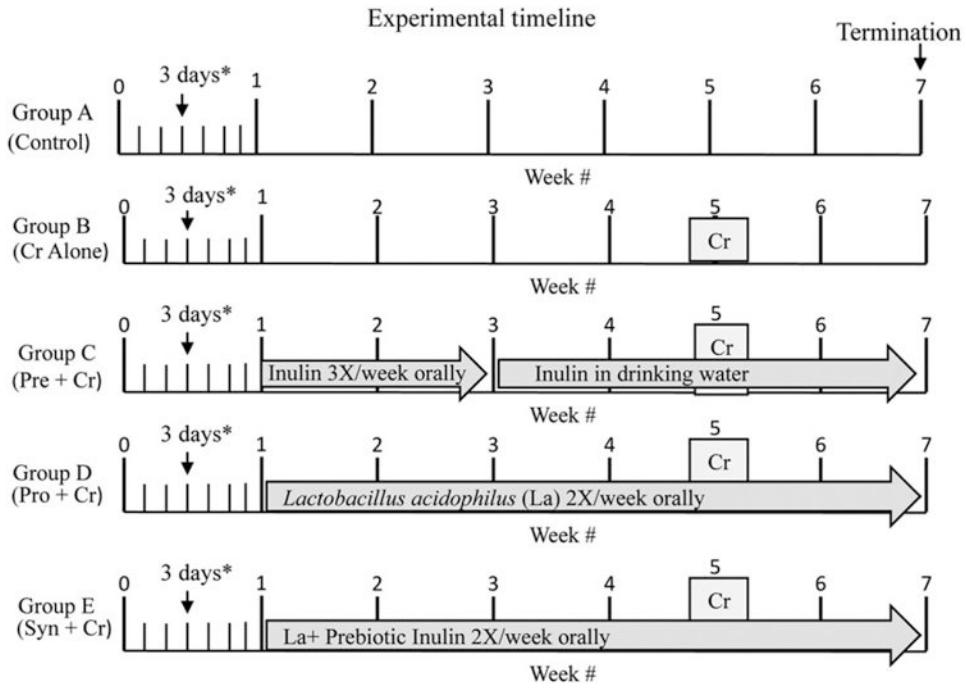

Fig. 1.

Experimental timeline. Three *in vivo* experiments were conducted in which 3-day-old mice along with lactating dams were randomly assigned to treatment groups of 7–10 mice pups per treatment. Group A (nontreated controls), group B (*Citrobacter rodentium* inoculated), group C (prebiotic inulin treated + *C. rodentium*), group D (probiotic *Lactobacillus acidophilus* + *C. rodentium*), group E (synbiotic combination probiotic *L. acidophilus* + prebiotic inulin + *C. rodentium*). Mice of treatment groups B, C, D, and E were inoculated with *C. rodentium* (Cr) at 5 weeks of age. All experimental procedures were terminated 2 weeks post-Cr pathogen inoculation.

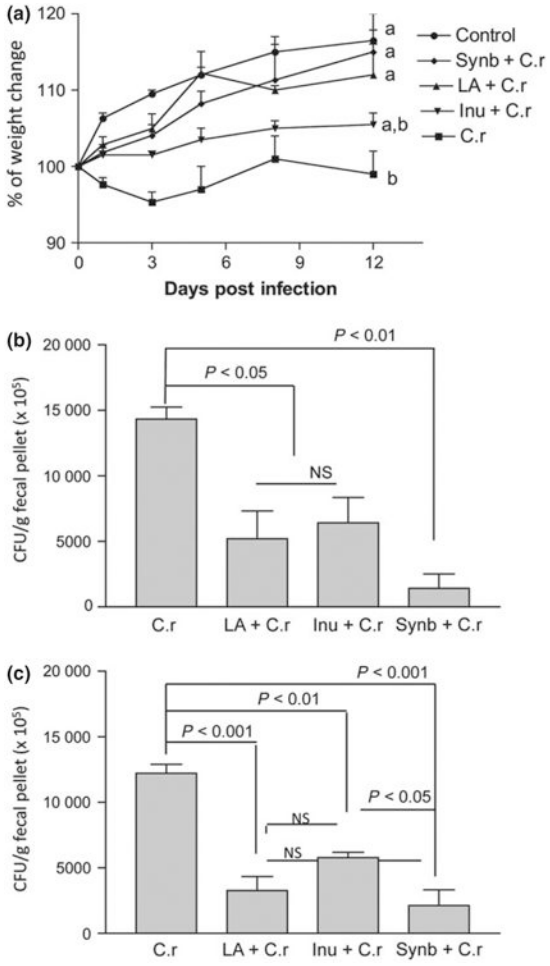

Fig. 2.

Early administration of probiotic *Lactobacillus acidophilus* (La) and/or prebiotic inulin reduces *Citrobacter rodentium* (Cr)-induced morbidity in mice. (a) Body weight changes of noninfected control mice (○) and mice pretreated with a combination of probiotic La and prebiotic (synbiotics ◆), probiotic La (▲), or prebiotic inulin (∇) and infected with *C. rodentium*, normal mice infected with *C. rodentium* alone (■) during the course of the experiments. Different letters represent significant difference ($P < 0.05$) based on one-way ANOVA (Tukey's multiple comparison test). (b and c) Mice that were pretreated with probiotic (La), prebiotic (inulin), and both and then infected with *C. rodentium* have significantly lower bacterial output in the fecal pellets at 1 and 2 weeks postinfection. The *C. rodentium* counts in fecal pellet were significantly higher in *C. rodentium*-infected group compared with the intermediate levels of bacteria recovery from mice pretreated with probiotic La or prebiotic inulin at 1 week ($P < 0.05$) or 2 weeks ($P < 0.01$ and $P < 0.001$, respectively) postinfection. The lowest level of bacterial output was detected in synbiotics group at 1 week ($P < 0.01$) and 2 week postinfection compared with *C. rodentium*-infected only group. Significant difference was also detected in the fecal bacterial output of in mice with symbiotic treatment compared with inulin-treated and *C. rodentium*-infected mice at 2

week postinfection ($P < 0.05$), based on one-way ANOVA (Tukey's multiple comparison test).

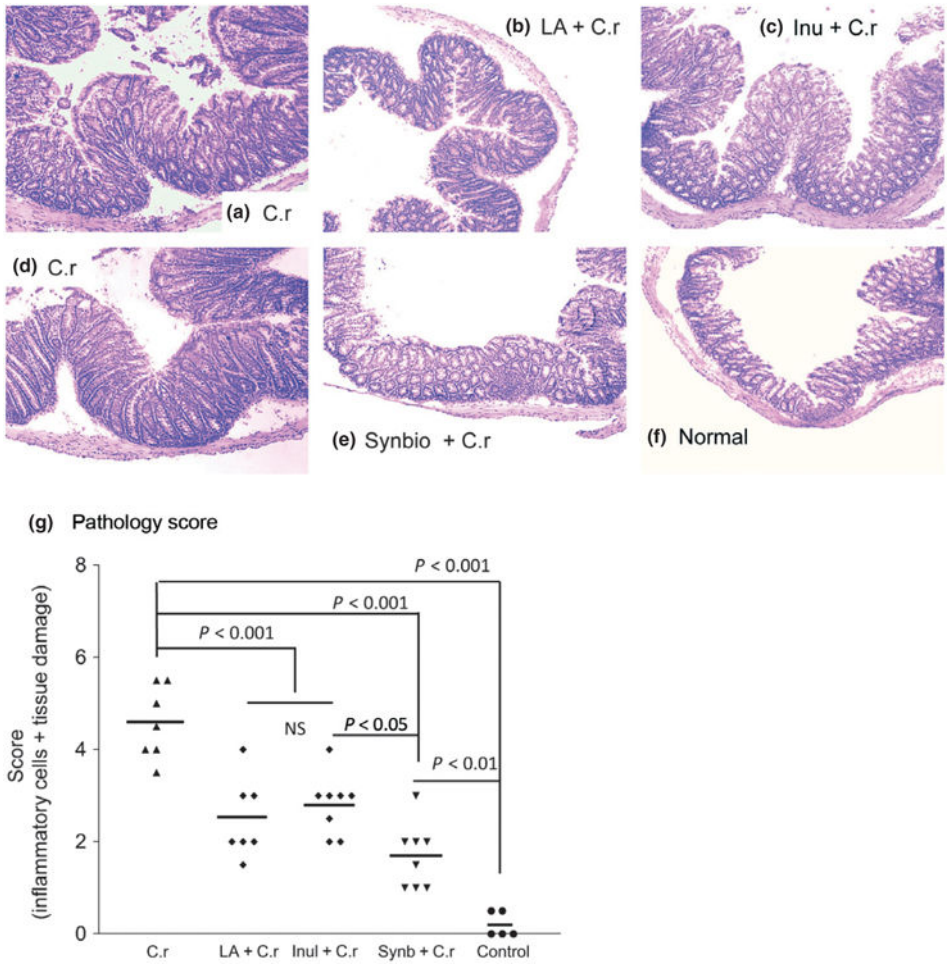

Fig. 3.

Representative histopathology of colonic tissues at 2 weeks post-Cr infection. Three independent experiments showing similar results with ≈ 7–10 mice per treatment. (a and d) Mice that were infected with *Citrobacter rodentium* only. (b) Mice pretreated with probiotic La and then infected with Cr. (c) Mice pretreated with prebiotic inulin and then infected with Cr. (e) Mice pretreated with synbiotic and then infected with Cr. (f) Normal mice. (g) The colonic pathology score of different groups of mice at 2 weeks after *C rodentium* infection. The scores were assessed by determination of inflammation and tissue damage. The figures shown are measurements of individual mice pooled from three independent experiments. The horizontal line represents the mean score of different groups. Data from the colonic pathology scores were analyzed using one-way analysis of variance (Tukey's multiple comparison test).

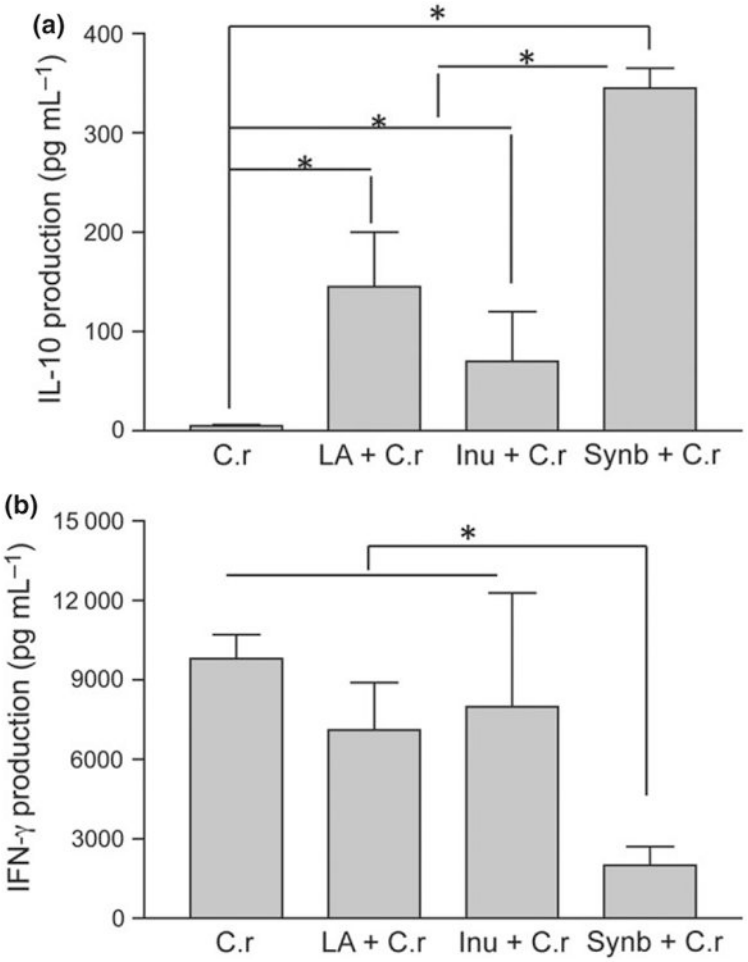

Fig. 4.

Pretreatment with probiotic *Lactobacillus acidophilus* (La), prebiotic inulin, or synbiotics alters cytokine responses in the MLN of mice. Cells from the MLN were isolated from mice 2 weeks post-*Citrobacter rodentium* infection of each treatment and exposed ex *vivo* to *C rodentium* antigen (C-Ag) in culture. Culture supernatants were collected after 72 h and assayed for cytokine production. (a) Synbiotic group showed a significant impact on the response of IL-10. (b) Pretreatment with synbiotic (probiotic La + prebiotic inulin) significantly reduced IFN-γ levels. **P* < 0.05 when compared to other treatment groups, based on one-way anova (Tukey's multiple comparison test). The results were displayed as means ± SEM and were representative of three independent experiments with *N* = 7–10 pups per treatment.

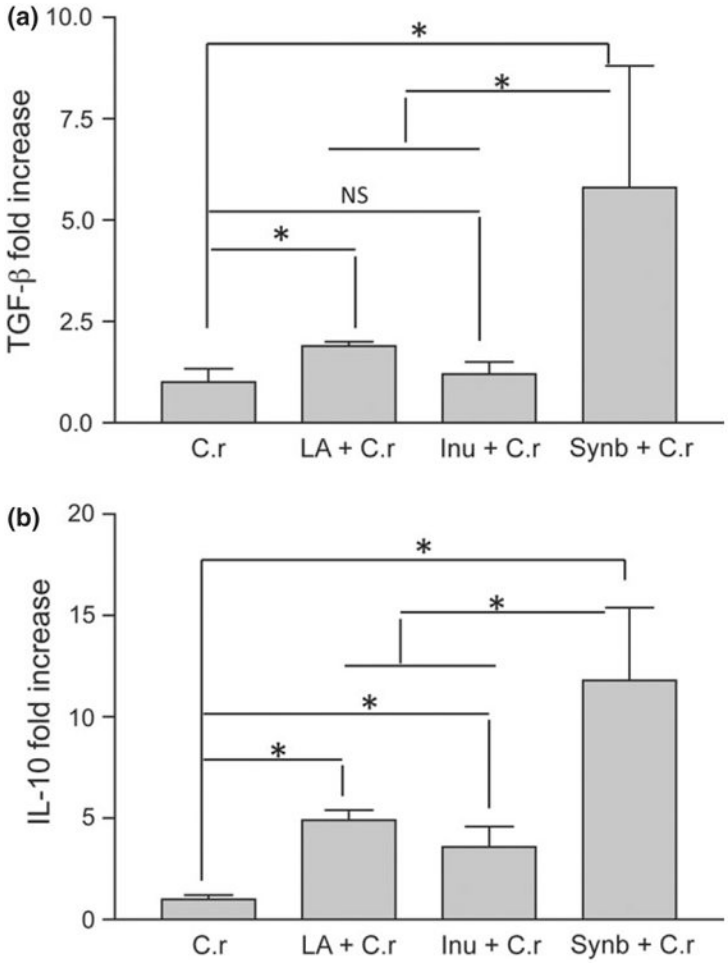

Fig. 5.

Probiotic (La) and synbiotic treatments promote regulatory cytokine expression in colonic tissue. Mice were pretreated with synbiotics (combination of probiotic La + prebiotic inulin), or probiotic La or prebiotic inulin and infected with *Citrobacter rodentium*. Cytokine mRNA expression in colon tissue was measured by real-time PCR at 2 weeks after Cr bacterial infection. Values represent the average fold change in mRNA expression in reference to the expression levels in Cr alone-infected mice with GAPDH mRNA expression as an internal control. (a) TGF-β response. (b) IL-10 response. *$P < 0.05$ when compared with that of Cr-infected group, based on one-way anova (Tukey's multiple comparison test). The results were displayed as means ± SEM and were representative of three independent experiments with $N = 7–10$ pups per treatment.

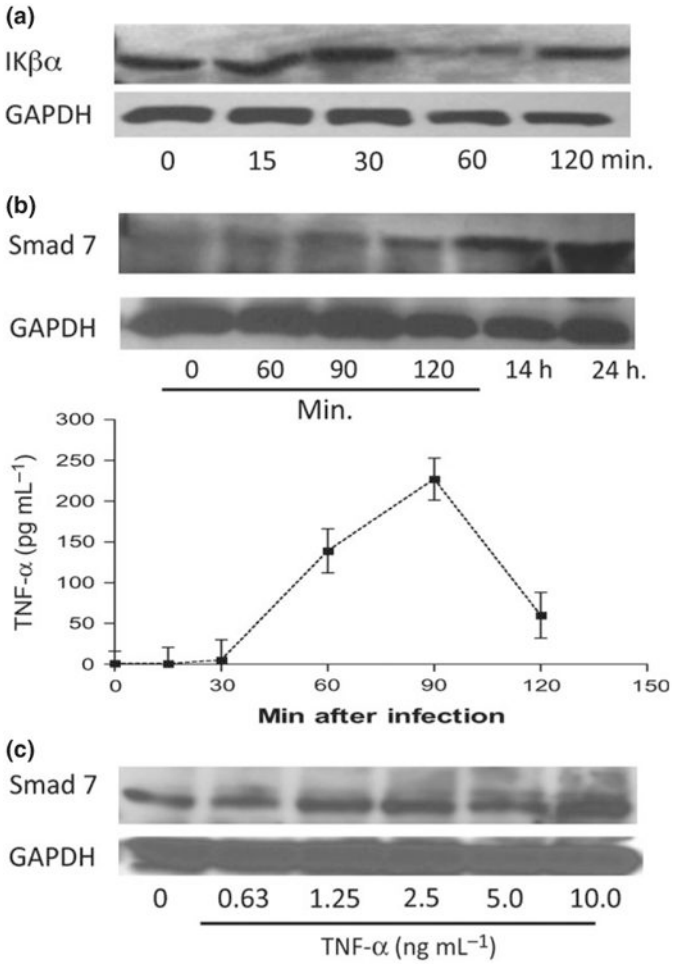

Fig. 6.

Activation of intracellular NF-kB and Smad 7 in mouse epithelial cells. (a) Confluent CMT93 (p23) cells were incubated with *Citrobacter rodentium* (Cr, 2.5×10^7 CFU per well) in a six-well plate for 1 h. Cells were subsequently washed in PBS and lysed. (a) Cell lysate (40 μg of protein per lane) was analyzed by Western blotting to determine temporal IkB-α degradation at 0, 15, 30, 60, and 120 min post-Cr pathogen exposure. (b) CMT93 (p25) cells were inoculated with Cr (2.5×10^7 CFU per well) in a six-well plate for 1 h, and 75 μg of protein per lane of cell lysate was analyzed by Western blotting at 0, 1, 1.5, 2, 14, and 24 h post-Cr pathogen exposure to determine temporal Smad 7 induction. Culture media was collected at each time point and assayed for TNF-α secretion (pg mL^{-1}) at each time point. (c) CMT93 cells (p28) were stimulated with TNF-α for 4 h in complete DMEM media, washed 2× in PBS, and 75 μg of protein/lane of cell lysate was analyzed by Western blotting to determine intracellular Smad 7 levels at various TNF-α doses. All data shown here are representative of nine independent *in vitro* experiments showing similar results.

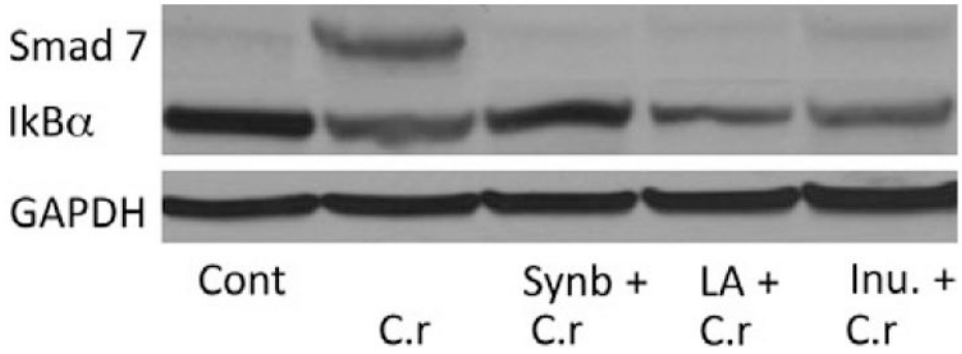

Fig. 7.

Probiotic La pretreatment attenuates Smad 7 and NF-kB activation *post-Citrobacter* rodentium exposure in the colon of mice pups. BALB/c ByJ mice were inoculated bi-weekly after birth with, probiotic La, prebiotic inulin, or a combination of both (synbiotic) and challenged with pathogen Cr at 5 weeks of age. Lysates from the colon were prepared 2 weeks post-Cr infection. Seventy-five micrograms of protein per lane of pooled lysates per treatment (≈ 7.5 μg to 10 μg of protein per mouse) from colonic tissue was analyzed by Western blotting to determine the effects of La, inulin, and synbiotic treatment, and Cr on Smad 7 and IkB-α signaling. Control mice only received a saline vehicle bi-weekly. The data shown here are the representative one of three *in vivo* experiments performed with *N*= 7–10 pups per treatment, showing similar results.