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Original Contribution

Lactobacillus rhamnosus blocks inflammatory signaling in vivo via reactive oxygen species generation

Patricia W. Lin [a,*], Loren E.S. Myers [a], Laurie Ray [a,b], Shuh-Chyung Song [a], Tala R. Nasr [a], Andrew J. Berardinelli [a,b], Kousik Kundu [c], Niren Murthy [c], Jason M. Hansen [d], Andrew S. Neish [b]

[a] Division of Neonatal–Perinatal Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA
[b] Division of Epithelial Pathobiology, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA
[c] Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA
[d] Division of Pulmonology, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA

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ABSTRACT

Uncontrolled inflammatory responses in the immature gut may play a role in the pathogenesis of many intestinal inflammatory syndromes that present in newborns or children, such as necrotizing enterocolitis (NEC), idiopathic inflammatory bowel diseases (IBD), or infectious enteritis. Consistent with previous reports that murine intestinal function matures over the first 3 weeks of life, we show that inflammatory signaling in the neonatal mouse gut increases during postnatal maturation, with peak responses occurring at 2–3 weeks. Probiotic bacteria can block inflammatory responses in cultured epithelia by inducing the generation of reactive oxygen species (ROS), which inhibit NF-κB activation through oxidative inactivation of the key regulatory enzyme Ubc12. We now report for the first time that the probiotic *Lactobacillus rhamnosus GG* (LGG) can induce ROS generation in intestinal epithelia in vitro and in vivo. Intestines from immature mice gavage fed LGG exhibited increased GSH oxidation and cullin-1 deneddylation, reflecting local ROS generation and its resultant Ubc12 inactivation, respectively. Furthermore, prefeeding LGG prevented TNF-α-induced intestinal NF-κB activation. These studies indicate that LGG can reduce inflammatory signaling in immature intestines by inducing local ROS generation and may be a mechanism by which probiotic bacteria can prevent NEC in premature infants or reduce the severity of IBD in children.

Uncontrolled inflammatory responses in the immature gut may play a role in the pathogenesis of many intestinal inflammatory syndromes that present in newborns or children, such as necrotizing enterocolitis (NEC) [1,2]; idiopathic inflammatory bowel diseases (IBD) [3], including Crohn disease (CD) or ulcerative colitis (UC); or infectious enteritis. Multiple investigators have documented inappropriately exaggerated inflammatory responses in immature intestinal epithelia [4–6] and abnormal intestinal bacterial colonization may trigger or exacerbate these responses [7,8]. Indeed, recent studies suggest that abnormal bacterial colonization in premature infants due to prolonged antibiotic administration may increase the risk of NEC [9,10], and altered microbial composition is thought to play a key role in the pathogenesis of IBD [3].

Probiotics are live microbes that are ingested to "exert health benefits beyond basic nutrition" [11]. Recently, probiotics containing mixtures of *Lactobacillus* and *Bifidobacterium* species have been shown to reduce the incidence and severity of NEC [12]. To elucidate the mechanism by which probiotic bacteria may prevent uncontrolled inflammatory responses implicated in NEC or IBD (CD, UC), we modeled immature intestinal epithelia both in vitro (FHs74Int) and in vivo (neonatal mice). Consistent with previous reports that murine intestinal function matures over the first 3 weeks of life [13], we show that inflammatory signaling in neonatal mouse intestines increases during postnatal maturation, with peak responses occurring at 2–3 weeks. Exuberant inflammatory responses in 2-week-old mice may reflect a propensity toward exaggerated inflammatory responses thought to occur in immature human intestines during their developmental window of NEC susceptibility [14,15]. We have previously shown that probiotic bacteria can block inflammatory responses in model intestinal epithelia by inducing local generation of reactive oxygen species (ROS) [16]. Oxidative stress has been implicated in many diseases affecting premature infants, including retinopathy of prematurity, chronic lung disease, intraventricular hemorrhage, and NEC. However, clinical studies administering antioxidants to premature infants have been disappointing [17–19].

Abbreviations: CD, Crohn disease; Cul1, cullin-1; DAPI, 4′,6-diamidino-2-phenylindole; DCF, 2′,7′-dichlorodihydrofluorescein diacetate; GSH, glutathione; GSSG, glutathione disulfide; IBD, idiopathic inflammatory bowel disease; LGG, *Lactobacillus rhamnosus GG*; NEC, necrotizing enterocolitis; NF-κB, nuclear factor κB; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α; UC, ulcerative colitis.

* Corresponding author. Fax: +1 404 727 8538.
E-mail address: pllin@emory.edu (P.W. Lin).

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This is probably because global ROS suppression may have negative effects on *physiologic* ROS signaling, which regulates many necessary, homeostatic processes [20]. ROS signaling has been implicated in regulating developmental processes in the fetus and premature newborn and depends on tightly regulated changes in cellular localization and concentration [21,22]. One mechanism by which ROS can regulate cellular processes is through transient oxidative inactivation of catalytic cysteine residues on key regulatory enzymes. By influencing these enzymes, ROS can regulate apoptotic, proliferative, and inflammatory signaling [23].

Specifically, in model intestinal epithelia, ROS have been shown to reduce inflammatory signaling through oxidative inactivation of Ubc12, a key enzyme regulating NF-κB activation. Ubc12 is responsible for activation of the specific ubiquitin ligase complex SCF-β^{TRCP} through neddylation of its cullin-1 (Cul1) subunit [16]. When Cul1 remains deneddylated, SCF-β^{TRCP} fails to ubiquitinate the inhibitor of NF-κB (IκB-α), a modification that normally targets IκB-α for proteasomal degradation [24]. NF-κB thus remains trapped in the cytosol by IκB-α, unable to translocate to the nucleus to activate transcription of inflammatory mediators. However, whether ROS signaling can regulate inflammatory signaling in an in vivo model or in immature intestinal epithelia is unknown.

Probiotics are composed of commensal bacteria that have been shown to improve intestinal host defenses through regulation of barrier function, proliferation, apoptosis, and inflammation [25–31]. Of the various commensal bacteria studied, *Lactobacillus rhamnosus GG* (LGG) is thought to be one of the most effective inducers of ROS and anti-inflammatory effects in cultured epithelial models [16]. LGG has also been shown to reduce inflammatory signaling in neonatal rats [32,33]. However, the mechanisms involved have not been fully elucidated. Here, we report for the first time that LGG can induce ROS and prevent inflammatory signaling in both in vitro and in vivo models of immature intestinal epithelia. Model immature intestinal epithelia (FHs74Int) exposed to LGG exhibited increased 2′,7′-dichlorodihydrofluorescein diacetate (DCF) fluorescence and reduced Cul1 neddylation, reflecting local ROS generation and its resultant Ubc12 inactivation, respectively. To confirm in vivo the relevance of these findings, we investigated the effects of LGG on intestinal epithelial ROS and inflammatory signaling when gavage fed to 2-week-old preweaned neonatal mice. As expected, intestines from immature mice gavage fed LGG exhibited increased epithelial ROS as detected by hydrocyanine-3 fluorescence, GSH oxidation, and Cul1 deneddylation. Furthermore, using a previously reported model of intestinal inflammation [34], we demonstrated that LGG could prevent intestinal NF-κB activation when prefed to immature mice. These studies indicate that LGG can reduce inflammatory signaling in immature intestines by inducing local ROS generation and may be a mechanism by which probiotic bacteria can prevent NEC in premature infants or reduce the severity of IBD or infectious enteritis in children.

Materials and methods

Cell and bacterial culture

Human fetal intestinal epithelial cells derived from 3-to 4-month gestation fetuses (FHs74Int from ATCC CCL-241) were grown to confluence in 0.69-cm^2 eight-well chamber slides (BD Biosciences, Bedford, MA, USA) or 9.5-cm^2 six-well plates (Corning Costar, Lowell, MA, USA) per the ATCC guidelines. Wild-type *Salmonella typhimurium* (SL3201) was maintained and prepared for use under nonagitated microaerophilic conditions as we have previously described [35]. LGG (from the ATCC, Manassas, VA, USA) was grown overnight, washed, and concentrated in PBS or medium as previously described [28]. LGG was applied to cells at 4×10^8 to 10^9 CFU or gavage fed to 2-week-old neonatal mice at 2×10^9 CFU.

In vitro experiments

FHs74Int cells were treated with medium with or without LGG for 30 min. Cells were viewed by fluorescence microscopy or prepared for Western blot analysis by scraping into ice-cold lysis buffer.

Animal care

C57BL/6J mice were bred at an animal facility at Emory University and all studies were approved by the Institutional Animal Care and Use Committee. For ex vivo infection, timed-pregnant C57BL/6J mice were used to allow accurate dating to prenatal and postnatal days −1 (E18), +2 to+8 days,+2 weeks, and+3 weeks. Mice were anesthetized with CO_2 and euthanized by cervical dislocation. Small intestines were subsequently isolated for ex vivo infection. For probiotic treatment, preweaned 2-week-old neonatal mice were gavage fed 2×10^9 CFU of LGG or carrier control for the times indicated. Mice were anesthetized with CO_2 and euthanized by cervical dislocation. Distal small intestinal sections were isolated for ex vivo infection and frozen in embedding medium (Sakura Finetek, Torrance, CA, USA) (for histologic staining) or small intestinal epithelial cells were scraped into ice-cold perchloric acid (PCA) solution (for GSH assay) or ice-cold RIPA lysis buffer (for cullin-1 Western blot analysis). To study inflammatory signaling in vivo, we treated (LGG or carrier prefed) mice with intraperitoneal 2.8 μg TNF-α (PeproTech, Rocky Hill, NJ, USA) or carrier control. (Claud et al. have previously reported that intraperitoneal injection of TNF-α induces intestinal epithelial activation of NF-κB within 90 min in immature mice [34].) Two hours later, distal small intestinal sections were collected into hypotonic buffer (supplemented with detergent and 1 mM dithiothreitol) provided by the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) and snap frozen for later analysis by NF-κB DNA binding ELISA.

Ex vivo intestinal infection model

Small intestinal sections were isolated and maintained ex vivo as previously described [28]. Previous studies have reported successful maintenance of murine intestinal organ culture for measurement of cytokine secretion [36]. To induce inflammatory cytokine release, we cultured small intestinal sections in warm RPMI with or without wild-type *S. typhimurium* for 2 h at 37°C. Culture supernatants were collected, centrifuged at 2000 g for 60 s (to remove bacteria and debris), and assayed for TNF-α secretion by ELISA (R and D Systems) according to the manufacturer's guidelines.

Histologic staining for ROS detection

For in vitro H_2O_2, confluent FHs74Int cells were loaded with 5 μM permeative DCF for 30 min, washed, and subsequently treated with or without LGG for an additional 30 min. Nuclei were subsequently counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were viewed by fluorescence microscopy (40× objective); the number of DCF-positive cells was counted and expressed as a percentage of total cells counted. For in vivo superoxide radicals, cryostat sections (6 μm) of fresh-frozen distal murine small intestines were incubated with 10 μM hydrocyanine (hydro-Cy3) for 1 h at 37°C and washed and the nuclei were subsequently counterstained with To-Pro-3 (Invitrogen, San Diego, CA, USA). Hydro-Cy3 was kindly provided by Dr. Niren Murthy (Georgia Institute of Technology, Atlanta, GA, USA). Images were viewed using a Zeiss LSM 510 confocal microscope (10× objective). Relative fluorescence was determined by quantitative digital analysis via FluoView (Olympus Corp., Melville, NY, USA).

GSH assay

To assay the GSH antioxidant pool, we measured GSH and GSSG concentrations by HPLC as *S*-carboxymethyl, *N*-dansyl derivatives

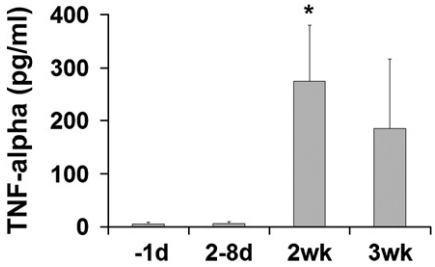

Fig. 1. Inflammatory signaling peaks at 2 weeks in immature murine intestinal organ culture. TNF-α secretion induced from murine small intestinal organ culture (over the first 3 weeks of life) treated with *S. typhimurium* ex vivo. Data are means ± SEM from at least four experimental repeats per condition. *P<0.05 compared to the −1 day condition.

using γ-glutamylglutamate as an internal standard as previously described [37]. Distal small intestines were immediately placed in cold 5% PCA buffer containing γ-glutamylglutamate. The epithelium of each prepared tissue was scraped and collected, sonicated, and centrifuged to remove debris. Samples were subsequently derivatized and analyzed by HPLC, and the intracellular GSH and GSSG levels measured were used in the Nernst equation to determine the redox potential for this thiol pair.

Western blot

For Western blot analysis of neddylated cullin-1, FHs74Int cells were collected in ice-cold SDS lysis buffer or murine intestinal epithelial cells were scraped into ice-cold RIPA lysis buffer containing protease inhibitor, sonicated, and centrifuged to remove debris. Samples were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose by electroblotting. Membranes were probed with anti-cullin-1 antibody (Zymed, Carlsbad, CA, USA) and HRP-conjugated anti-rabbit IgG (GE Healthcare, Chalfont St. Giles, UK) as previously described [16]. Equal amounts of protein were loaded in each lane as determined by the Bradford protein assay. Band densitometry was measured by Adobe Photoshop.

DNA binding ELISA for activated NF-κB

Samples were thawed on ice and then homogenized and processed per kit instructions to produce the cytoplasmic and nuclear extracts. Protein concentrations were determined by the Bradford protein assay. The amount of activated NF-κB was quantified by DNA binding ELISA (TransAM transcription factor assay; Active Motif) according to the manufacturer's guidelines. The TransAM p65 NF-κB activation assay specifically measures binding of activated NF-κB to its consensus site (5′-GGGACTTTCC-3′). The amount of bound NF-κB is detected by primary antibody to the p65 subunit of the p50/p65 heterodimer. The protein concentration was normalized for each sample to 2 μg for this ELISA-based assay. Results were visualized using the provided chemiluminescence reagents on a Synergy HT multimode microplate reader (BioTek, Winooski, VT, USA).

Statistical analysis

Statistical differences were determined by one-way ANOVA or Student's t test. A $P<$ 0.05 was considered statistically significant.

Results

Inflammatory signaling peaks at 2 weeks in the developing murine intestine

Intestinal epithelial architecture and barrier function are known to develop postnatally in the neonatal mouse, with maturity expected at 3 weeks [13]. Therefore, 2-week-old mice have often been used to

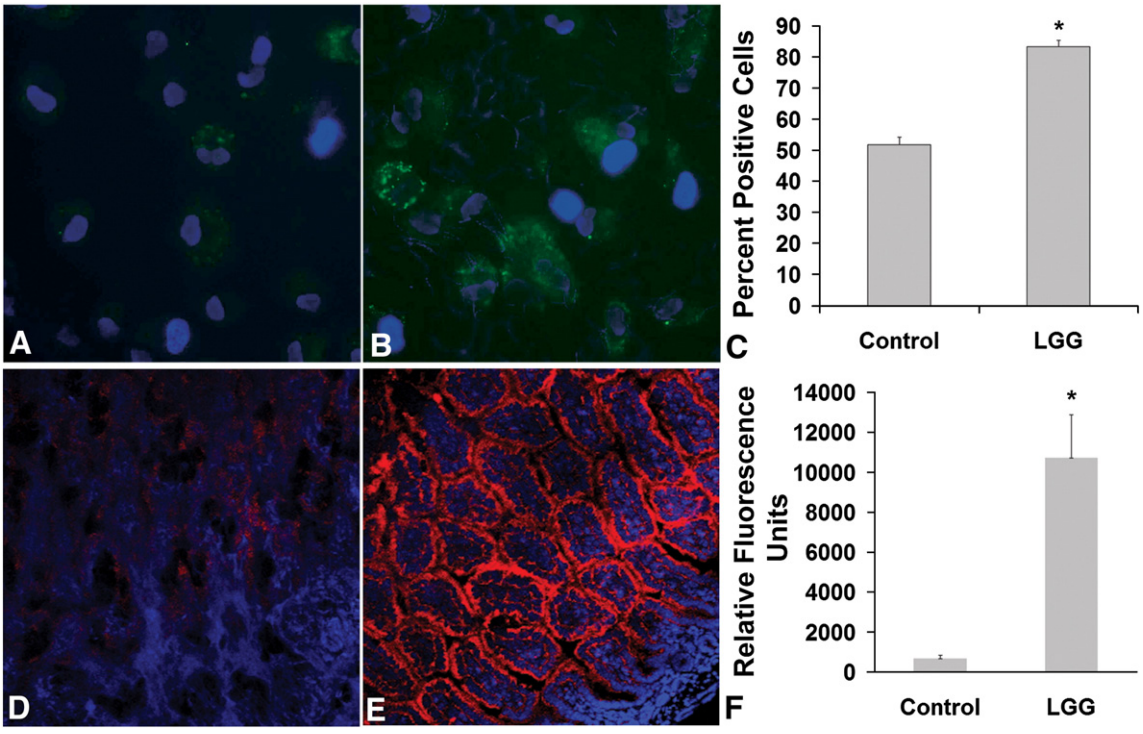

Fig. 2. LGG induces ROS generation in immature intestinal epithelia. (A and B) DCF staining (green) in confluent FHs74Int cells treated with (B) or without (A) LGG for 30 min. Nuclei are counterstained with DAPI (blue). (C) Average percentage (mean ± SEM) of DCF-positive cells per treatment condition as indicated (n = 3 experiments). (D and E) Hydro-Cy3 (red) staining in distal small intestines isolated from immature mice prefed LGG (E) or carrier control (D). Murine intestinal epithelial nuclei are counterstained with To-Pro-3 (blue). (F) Quantification of ROS detection as determined by relative Cy3 fluorescence per high-power field per treatment condition as indicated. Data are means ± SEM (representative of three independent experiments). *P<0.05 compared to control condition.

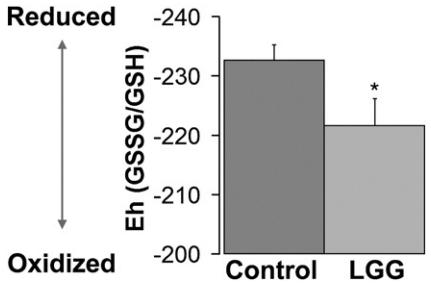

Fig. 3. LGG induces GSH oxidation in immature intestinal epithelia. The GSSG/GSH redox potential of distal small intestinal epithelia isolated from immature mice 1 h after being fed LGG or carrier control is shown. *$P<0.05$ compared to control condition.

model premature human intestines [28,34,38]. Previous reports indicate that 2-week-old immature rodent intestines exhibit exaggerated inflammatory responses compared to adult rodent intestines [4,34]. However, the developmental timeline of this inflammatory response has not been characterized. Thus, we measured inflammatory responses over the first 3 weeks of postnatal murine life in an ex vivo model of intestinal infection. Older murine intestines demonstrated robust proinflammatory responses as measured by increased TNF-α secretion, with 2-week-old ex vivo intestines demonstrating the strongest responses (Fig. 1). These results support the idea that murine intestines exhibit maximal vulnerability to exaggerated inflammatory responses at 2 weeks of life.

LGG induces ROS generation in immature intestinal epithelia

Delayed or inappropriate intestinal bacterial colonization has been implicated in the pathogenesis of NEC [1,9,10,39]. Probiotic administration may prevent NEC [12] by normalizing bacterial populations and consequently reducing inflammatory signaling. We have previously shown that probiotic bacteria can block inflammatory responses through ROS signaling in cultured epithelia [16]. To determine whether the probiotic LGG can reduce inflammatory signaling in our in vivo murine model for premature intestines, we measured the effects of LGG on epithelial ROS generation, GSH oxidation, and Cul1 deneddylation.

We first measured ROS using the membrane-permeative DCF in an in vitro model of immature intestinal epithelial using a primary intestinal epithelial cell line isolated from 3-to 4-month-old human fetuses. Increased cytoplasmic ROS was detected in cultured immature intestinal epithelia within 30 min of exposure to LGG (Figs. 2A–2C). To measure transient and subtle changes in immature intestinal epithelial ROS in vivo, we employed hydrocyanines, a new family of fluorescent probes known to be more stable and sensitive than traditional ROS probes [40], and our murine model of immature intestine [28]. Hydrocyanines can detect both superoxide anions and hydroxyl radicals, whereas DCF detects hydrogen peroxide species. We have previously reported that gavage-fed material can reach the colon within 30 min in immature mice and that LGG specifically can be recultured from the intestines of immature mice up to 4 h after gavage feeding [28]. Intestines isolated from mice gavage fed LGG exhibited rapidly increased (by 30 min) epithelial ROS generation as detected by hydro-Cy3 staining (Figs. 2D–2F). These results indicate that LGG can induce ROS generation in immature small intestinal epithelia both in vitro and in vivo.

LGG induces GSH oxidation in immature intestinal epithelia

To confirm the significance of these results, we assayed GSH, a major antioxidant system involved in peroxide elimination. Changes in the ratio of GSH to GSSG and GSH redox status are commonly used as an indicator of oxidative stress and changes in the redox potential suggest changes in ROS production in the intracellular environment. We fed immature mice medium with or without LGG for 60 min. This time point is consistent with previous ileal loop studies, which measured intestinal ROS production within 30 min of bacterial exposure [16]. Immature intestines isolated from mice fed LGG exhibited increased oxidation of GSH compared to intestines isolated from control mice (Fig. 3), indicating that these immature intestines may be responding to a new oxidant stimulus.

LGG prevents neddylation of cullin-1 in immature intestinal epithelia

To determine whether LGG can prevent inflammatory signaling through oxidative inactivation of Ubc12, we measured the effects of LGG exposure on Cul1 neddylation in both our in vitro and in vivo models of immature intestinal epithelia by Western blot. Because Ubc12 is responsible for activation of the ubiquitin ligase complex, SCF-β^{TRCP}, through neddylation of its Cul1 subunit [16], the increased presence of Cul1 in its deneddylated form indicates Ubc12 inacti-

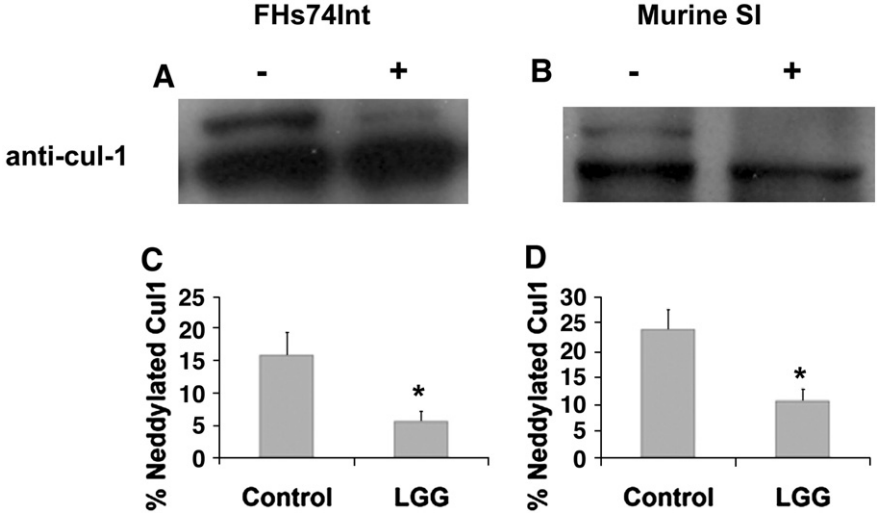

Fig. 4. LGG induces cullin-1 deneddylation in immature intestinal epithelia. (A and B). Cul1 Western blots of confluent FHs74Int cells (A) or of epithelia isolated from immature preweaned distal small intestines (B). FHs74Int cells were collected 30 min after treatment with LGG or carrier control. Small intestinal epithelia were isolated 1 h after gavage feeding with LGG or carrier control. (C and D) Quantification of percentage neddylated Cul1 as determined by band density analysis of three representative Western blots. Data are represented as means ± SEM. *$P<0.05$ when compared to control condition.

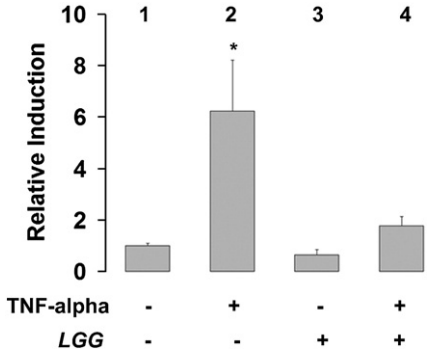

Fig. 5. LGG prevents TNF-α-induced NF-κB activation in immature intestines. Activated NF-κB was measured by DNA binding ELISA from the nuclear extracts of distal small intestines isolated from control immature mice prefed medium with (column 3) or without (column 1) LGG (1 h before intraperitoneal PBS) or from immature mice stimulated with intraperitoneal TNF-α 1 h after prefeeding of medium with (column 2) or without (column 4) LGG. Each bar represents the average fold induction of NF-κB activation over control (medium-fed, PBS-injected mice). Data are means ± SEM, $n = 4$ experiments. *$P < 0.05$ compared to all other treatment groups.

vation. Failure to activate SCF-β^{TRCP} ultimately results in failure to ubiquitinate and degrade the inhibitor of NF-κB (IκB-α), thus preventing inflammatory signaling. Confluent model immature intestinal epithelia (FHs74Int) exposed to LGG demonstrated deneddylation of Cul1 within 30 min of exposure (Fig. 4A). Similarly, small intestinal epithelia obtained from mice gavage fed LGG showed increased Cul1 deneddylation compared to mice fed vehicle control. These results indicate that LGG can induce oxidative inactivation of key regulatory enzymes responsible for inflammatory signaling.

LGG blocks NF-κB activation in immature intestines

Next we tested whether LGG can reduce inflammatory signaling in an in vivo murine model of immature intestines, which has been previously reported to exhibit exaggerated inflammatory responses compared to mature intestines. Claud et al. have previously demonstrated that intraperitoneal TNF-α induces intestinal epithelial NF-κB activation within 90 min [34]. To demonstrate that induction of Cul1 deneddylation by LGG ultimately results in blockade of NF-κB activation, we compared NF-κB activation in the small intestines of immature mice fed with or without LGG before TNF-α activation. As expected, intraperitoneal TNF-α induced a sixfold increase in intestinal activated (nuclear) NF-κB compared to vehicle control (Fig. 5, compare columns 1 and 3). However, if immature mice were prefed LGG, TNF-α injection failed to induce increased intestinal nuclear NF-κB (Fig. 5, compare columns 3 and 4), suggesting that LGG can indeed block inflammatory signaling through NF-κB in the intestines of immature mice. LGG alone had no effect on NF-κB activation (Fig. 5, columns 1 and 2), indicating, as expected, that this commensal has no intrinsic proinflammatory activity.

Discussion

Uncontrolled intestinal inflammatory responses have been implicated in the pathophysiology of many intestinal inflammatory syndromes such as infectious enteritis, IBD (CD and UC), and NEC [1–3]. Thus, investigations aimed at understanding and mitigating these exuberant inflammatory responses is a stated priority [39]. Recent clinical [12,41–45] and animal [46–50] studies have demonstrated that probiotic bacteria may be a particularly promising preventive therapy for reducing the incidence and severity of NEC. Despite this, oral administration of live bacteria to the immunocompromised population of very low birth weight infants most at risk for this disease remains a real concern. Thus, studies aimed at understanding the mechanisms of probiotic-induced beneficial effects on immature intestines are needed so that targeted therapies that carry less infectious risk can be developed. We show in this study that the probiotic bacterium LGG may reduce inflammatory responses in immature intestines by inducing local epithelial ROS.

Epidemiologic studies indicate that NEC presents at around 32 weeks postconceptual age regardless of gestational age at birth, implicating a developmental period of susceptibility [14,51]. Both inappropriate inflammatory responses and abnormal intestinal bacterial colonization may play a role in the timing of peak susceptibility to NEC in premature infants. Multiple investigators have demonstrated that immature human intestinal epithelia exhibit exaggerated inflammatory responses [4–6] and thus the developmental susceptibility to NEC may be due to developmental changes in the intestinal epithelial inflammatory response to luminal contents. Murine intestinal epithelial architecture and function are known to be immature at birth compared to human intestinal epithelia, with epithelial function expected to mature by 3 weeks [13]. Murine intestinal immune development is also immature compared to human intestines, with lymphoid clusters evident before birth in humans but not until 7–10 days postnatally in mice [13]. Previous authors have reported exaggerated intestinal epithelial inflammatory responses in 2-week-old rodents compared to adult [4,34]. Here, we show for the first time that murine intestinal epithelia exhibit a developmental peak in inflammatory responses at 2 weeks of age. These data indicate that 2-week-old murine intestines may be an ideal model for the exuberant premature intestinal inflammatory response thought to be crucial to the pathogenesis of NEC.

Maturation of intestinal mucosal immunity and gut-associated lymphoid tissue (GALT) depends on intestinal colonization with commensal bacteria [52]. Premature human intestines and preweaned murine intestines are similar in that mucosal immunity and GALT are maturing postnatally [13,52]. The developmental window for the onset of NEC in premature infants may occur as the immature intestine tries to negotiate these maturational changes simultaneous with bacterial colonization. An undesirable exaggerated inflammatory response leading to further intestinal injury and profound systemic illness observed during severe NEC may be the result. Abnormal bacterial colonization may trigger or exacerbate these processes [7,8]. In fact, as molecular techniques emerge to improve characterization of intestinal colonization in the premature neonate, evidence accumulates that preterm human intestines indeed exhibit both delayed and abnormal bacterial colonization [53]. *Lactobacillus* species, in particular, have been shown to colonize later and less effectively and to be more susceptible to further reduced populations during antibiotic treatment or times of stress [53]. This is particularly concerning in light of our previous reports that *Lactobacillus* species are the most effective commensal species in mitigating inflammatory responses [16,27]. Because commensal bacteria are clearly important for growth, maturation, and cytoprotection of the intestine [25, 54], probiotics may act to prevent NEC in premature infants both by directly improving intestinal immune function and by normalizing bacterial populations.

Excessive ROS generation causes oxidative stress, which has been implicated in many disease processes [20,55–57]. Fetal development occurs in a relatively hypoxic environment. Premature infants are thought to be particularly vulnerable to oxidative stress because they have immature antioxidant regulation systems and are suddenly exposed to a relatively hyperoxic extrauterine environment at birth [22]. Based on animal models of NEC that model hypoxic–ischemic intestinal injury in an immature gut, oxidative injury has been implicated in NEC pathogenesis. However, clinical studies administering antioxidants to premature infants have failed to show benefit [17–19], and recently the validity of these animal models in accurately recapitulating the early steps in the pathogenesis of human NEC has been questioned [15]. Although these animal models have been invaluable in characterizing the exuberant inflammatory response in

NEC, future studies aimed at understanding the developmental window of NEC susceptibility observed clinically are needed to better target potential preventive interventions in this unpredictable and devastating disease.

Emerging evidence indicates *physiologic* ROS signaling regulates many necessary, homeostatic processes, and therefore, global ROS inhibition may be undesirable [20]. ROS signaling has been implicated in regulating developmental processes in the fetus and premature newborn and depends upon tightly regulated changes in cellular localization and concentration [21,22]. Here we show that the probiotic commensal LGG can block activation of the classic proinflammatory transcription factor NF-κB in the distal small intestines of immature mice by inducing epithelial ROS generation and preventing Cul1 neddylation required for activation of the ubiquitin ligase complex. We have previously shown in vitro that these effects are mediated by transient oxidative inactivation of the neddylation enzyme Ubc12 [16]. Recent development of a small-molecule inhibitor of another NEDD8-activating enzyme with potential for clinical application indicates that this pathway may be specifically targeted by noninfectious pharmacological agents [58]. This is especially important given the continued concern for clinical use of live probiotic bacteria in the immunocompromised population of premature infants.

These data provide one potential mechanism for the beneficial effects seen when probiotics are administered to premature infants [12]. Interestingly, LGG failed to induce ROS generation in proximal immature intestinal epithelia (data not shown). This may be due to differences in intestinal epithelia redox potential or differences in intestinal epithelial bacterial responsiveness throughout the intestine. Bacterial colonization varies throughout the intestine, with the highest concentrations of bacteria occurring in the distal small intestine and ascending colon [59]. This may explain both the propensity for NEC to originate in the ileum and the increased responsiveness to commensal bacterial modulations in that area. Future studies characterizing the redox potential in the different cellular compartments throughout the intestine may elucidate the role of redox signaling in postnatal maturation of intestinal defenses and may allow a better understanding of the role of ROS in IBD. A specific optimal physiologic level of ROS may be necessary to prevent excessive NF-κB activation and downstream inflammatory signaling. However, excessive ROS could cause collateral damage through direct cytotoxic effects or undesirable suppression of NF-κB-activated cytoprotective effects. Future studies aimed at elucidating the role of commensal bacteria and ROS signaling on the postnatal maturation of the immature gut may aid in the development of targeted therapies to prevent or reduce the severity of NEC or other childhood intestinal inflammatory syndromes.

Acknowledgments

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