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

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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 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 severity of IBD in children.

Keywords

Necrotizing enterocolitis; inflammatory bowel disease; inflammation; reactive oxygen species; probiotics; lactobacillus

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Introduction

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's 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 which 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 the propensity towards exaggerated inflammatory responses thought to be occurring 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]. This is likely 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 upon 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 has 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-beta^{TRCP} through neddylation of its cullin-1 (Cul1) subunit[16]. When Cul1 remains deneddylated, SCF-beta^{TRCP} fails to ubiquitinate the inhibitor of NF-κB (IκB-α), a modification which 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 which 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 increased2′,7′ dichlorodihydrofluorescein diacetate (DCF) fluorescence and reduced Cul1 neddylation reflecting local ROS generation and its resultant Ubc12 inactivation, respectively. To confirm *in vivo* relevance of these findings, we investigated the effect 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 severity of IBD or infectious enteritis in children.

Materials and Methods

Cell and bacterial culture

Human fetal intestinal epithelial cells derived from 3-4 month gestation fetuses (FHs74Int from ATCC CCL-241) were grown to confluence in 0.69 cm^2 8-well chamber slides (BD Biosciences, Bedford, MA) or 9.5 cm^2 6 well plates (Corning Costar, Lowell, MA) per ATCC guidelines. Wild type *Salmonella typhimurium* (SL3201) was maintained and prepared for use under non-agitated microaerophilic conditions as we have previously described[35]. *LGG* (from ATCC) was grown overnight, washed, concentrated in PBS or media as previously described[28]. *LGG* was applied to cells at 4×10^8-10^9 CFUs or gavage fed to 2 week old neonatal mice at 2×10^9 CFUs.

In vitro experiments

FHs74Int cells were treated with media with or without *LGG* for 30 minutes. 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. *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. *Probiotic treatment:* Preweaned 2 week old neonatal mice were gavage fed 2×10^9 CFUs 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, frozen in embedding medium (Sakura Finetek, Torrance, CA) (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 2.8μg TNF-α (Peprotech, Rocky Hill) or carrier control. (Claud, et al., have previously reported that intraperitoneal injection of TNF-α induces intestinal epithelial activation of NF-κB within 90 minutes in immature mice[34].) Two hours later, distal small intestinal sections were collected into hypotonic buffer (supplemented with detergent and 1 mM DTT) provided by the Nuclear Extract Kit (Active Motif, Carlsbad, CA) 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 *Salmonella typhimurium* for 2 hours at 37°C. Culture supernatants were collected, centrifuged at 2000g for 60 seconds (to remove bacteria and debris), and assayed for TNF-α secretion by ELISA (R&D Systems) according to manufacturer's guidelines.

Histologic staining

ROS detection—(*In vitro* H_2O_2) Confluent FHs74Int cells were loaded with 5μM permeant 2′,7′ dichlorodihydrofluorescein diacetate or H_2DCFDA-AM (DCF) for 30 minutes, washed, and subsequently treated with or without *LGG* for an additional 30 minutes. Nuclei were subsequently counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were viewed by fluorescence microscopy (40x objective); number of DCF-positive cells were counted and expressed as a percentage of total cells counted. *(In vivo superoxide radicals)* Cryostat sections (6 μm) of fresh frozen distal murine small intestines were incubated with 10 μM hydrocyanine (hydro-Cy3) for 1h at 37°C, washed and nuclei subsequently counterstained with To-Pro-3 (Invitrogen, CA). Hydro-Cy3 was kindly provided by Dr. Niren Murthy (Georgia Institute of Technology, Atlanta, GA). Images were viewed using Zeiss LSM 510 confocal microscope (10x objective). Relative fluorescence was determined by quantitative digital analysis via FluoView (Olympus Corporation, Melville, NY).

GSH assay

To assay the GSH antioxidant pool, we measured GSH and GSSG concentrations by HPLC as Scarboxymethyl, N-dansyl derivatives using γ-glutamyl-gluatmate as an internal standard as previously described[37]. Distal small intestines were immediately placed in cold 5% PCA buffer containing γ-glutamyl-glutamate. The epithelium of each prepared tissue was scraped and collected, sonicated and centrifuged to remove debris. Samples were subsequently derivatized, analyzed by HPLC, and measured intracellular GSH and GSSG levels 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 by SDS polyacrylamide gels, and transferred to nitrocellulose by electroblotting. Membranes were probed with anti-cullin-1 antibody (Zymed, Carlsbad, CA) and HRP conjugated anti-Rabbit IgG (GE Healthcare, Chalfont St. Giles) as previously described[16]. Equal amounts of protein were loaded in each lane as determined by Bradford Protein Assay. Band densitometry was measured by Adobe Photoshop.

DNA Binding ELISA for activated NF-κB

Samples were thawed on ice, then homogenized and processed per kit instructions to produce the cytoplasmic and nuclear extracts. Protein concentrations were determined by the Bradford Protein Assay. Amount of activated NF-κB was quantified by DNA binding ELISA (TransAM Transcription Factor Assay, Active Motif) according to manufacturer's guidelines. TransAM p65 NF-κB activation assay specifically measures binding of activated NF-κB to its consensus site (5′-GGGACTTTCC-3′). Amount of bound NF-κB is detected by primary antibody to the p65 subunit of the p50/p65 heterodimer. Protein concentration was normalized for each sample to 2μg for this ELISA-based assay. Results were visualized using the provided

chemiluminescent reagents on a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT).

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 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 *Lactobacillus rhamnosus* (*LGG)* can reduce inflammatory signaling in our *in vivo* murine model for premature intestines, we measured the effect of *LGG* on epithelial ROS generation, GSH oxidation, and Cul1 deneddylation.

We first measured ROS using the membrane permeant 2′,7′ dichlorodihydrofluorescein diacetate (DCF) in an *in vitro* model of immature intestinal epithelial using a primary intestinal epithelial cell line isolated from 3-4 month-old human fetuses. Increased cytoplasmic ROS was detected in cultured immature intestinal epithelia within 30 minutes of exposure to *LGG* (Figs. 2A-C). In order 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, while DCF detects hydrogen peroxide species. We have previously reported that gavage fed material can reach the colon within 30 minutes in immature mice and that *LGG* specifically can be recultured from the intestines of immature mice after up to 4 hours after gavage feeding[28]. Intestines isolated from mice gavage fed *LGG* exhibited rapidly increased (by 30 minutes) epithelial ROS generation as detected by hydro-Cy3 staining (Fig 2D-F). 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 glutathione (GSH), a major antioxidant system involved in peroxide elimination. Changes in the ratio of GSH to GSSG and GSH redox

status is 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 media with or without *LGG* for 60 minutes. This time point is consistent with previous ileal loop studies, which measured intestinal ROS production within 30 minutes 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 effect of *LGG* exposure on Cul1 neddylation in both our *in vitro* and *in vivo* models of immature intestinal epithelia by Western blot. Since Ubc12 is responsible for activation of the ubiquitin ligase complex, SCF-beta^{TRCP} through neddylation of its Cul1 subunit[16], increased presence of Cul1 in its deneddylated form indicates Ubc12 inactivation. Failure to activate SCF-beta^{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 minutes 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 have 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 minutes[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* prior to TNF-α activation. As expected, intraperitoneal TNF-α induced a 6-fold increase in intestinal activated (nuclear) NF-κB when compared to vehicle control (Fig. 5, compare bars 1 and 3). However, if immature mice prefed *LGG*, TNF-α injection failed to induce increased intestinal nuclear NF-κB (Fig. 5, compare bars 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, bars 1 and 2), indicating, as expected, that this commensal has no intrinsic pro-inflammatory activity.

Discussion

Uncontrolled intestinal inflammatory responses have been implicated in the pathophysiology of many intestinal inflammatory syndromes such as infectious enteritis, IBD (CD or UC), or 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 prior to 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 when 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 upon 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 onset of NEC in premature infants may occur as the immature intestine tries to negotiate these maturational changes simultaneously 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, less effectively, and 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]. Since 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 relative 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 which 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]. While 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 non-infectious 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 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 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.

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List of Abbreviations

CD, Crohn’s 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 kappa B
ROS, reactive oxygen species
TNF-α, tumor necrosis factor alpha
UC, ulcerative colitis

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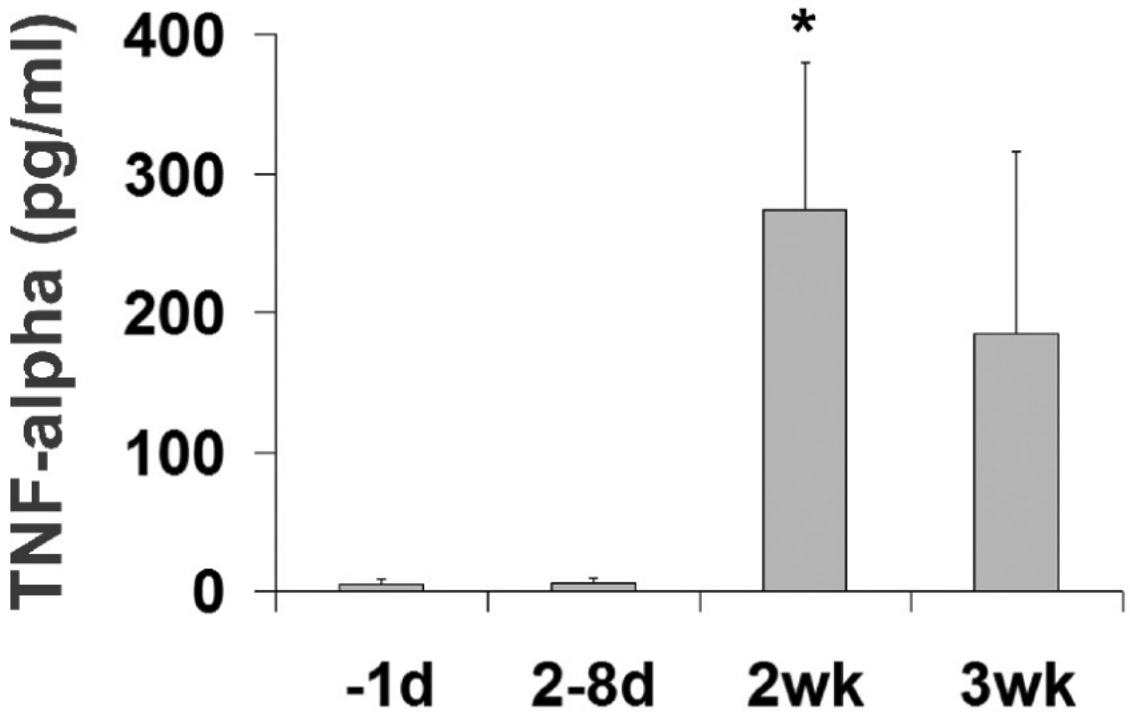

Figure 1. Inflammatory signaling peaks at 2 weeks in immature murine intestinal organ culture

TNF-α secretion induced from murine small intestinal organ culture (over the first 3 weeks of life) treated with *Salmonella typhimurium ex vivo*. Data are means ± SEM from at least 4 experimental repeats per condition. **P* < 0.05 when compared to -1d condition.

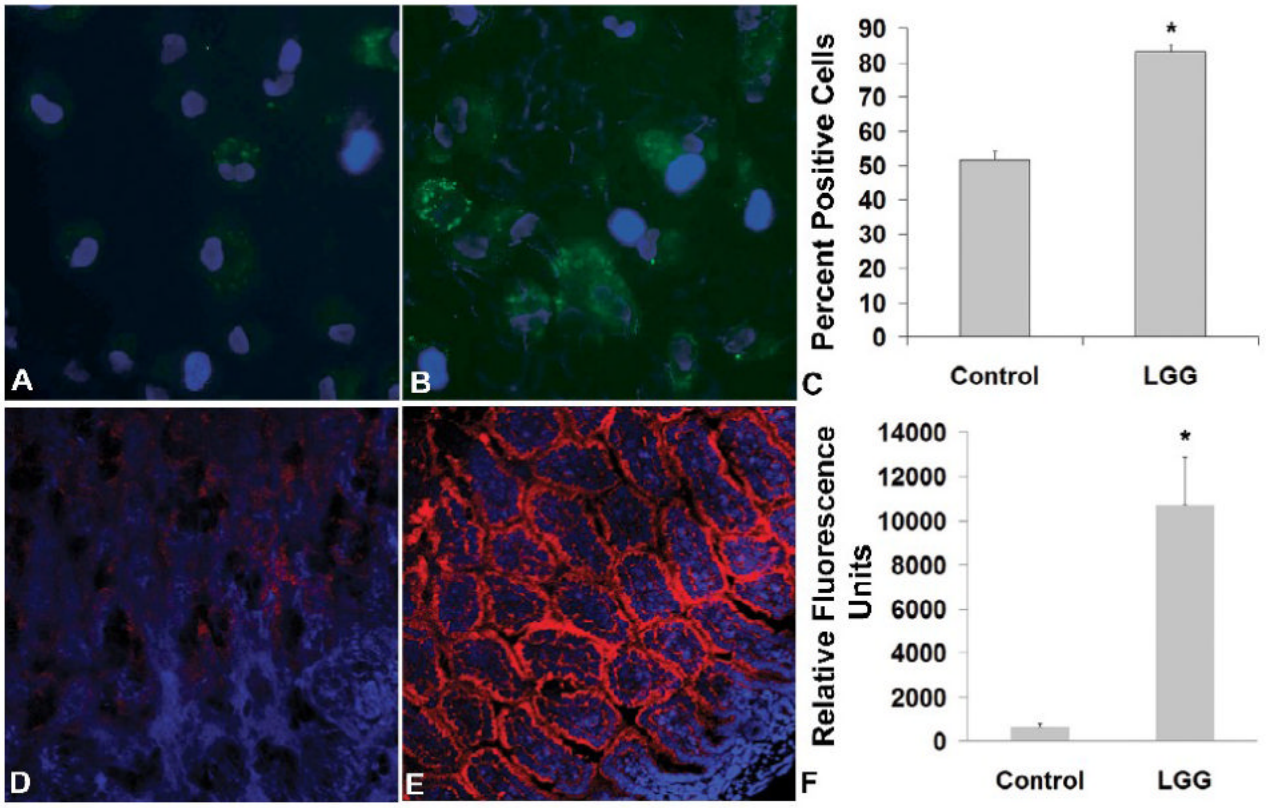

Figure 2. *LGG* induces ROS generation in immature intestinal epithelia

A-B, DCF staining (green) in confluent FHs74Int cells treated with *(B)* or without *(A) LGG* for 30 minutes. Nuclei counterstained with DAPI (blue). *C*, Average percent of (mean ± SEM) of DCF positive cells per treatment condition as indicated (n=3 experiments). *D-E*, Hydro-Cy3 (red) staining in distal small intestines isolated from immature mice prefed *LGG (E)* or carrier control *(D)*. Murine intestinal epithelial nuclei 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 3 independent experiments). *$P < 0.05$ when compared to control condition.

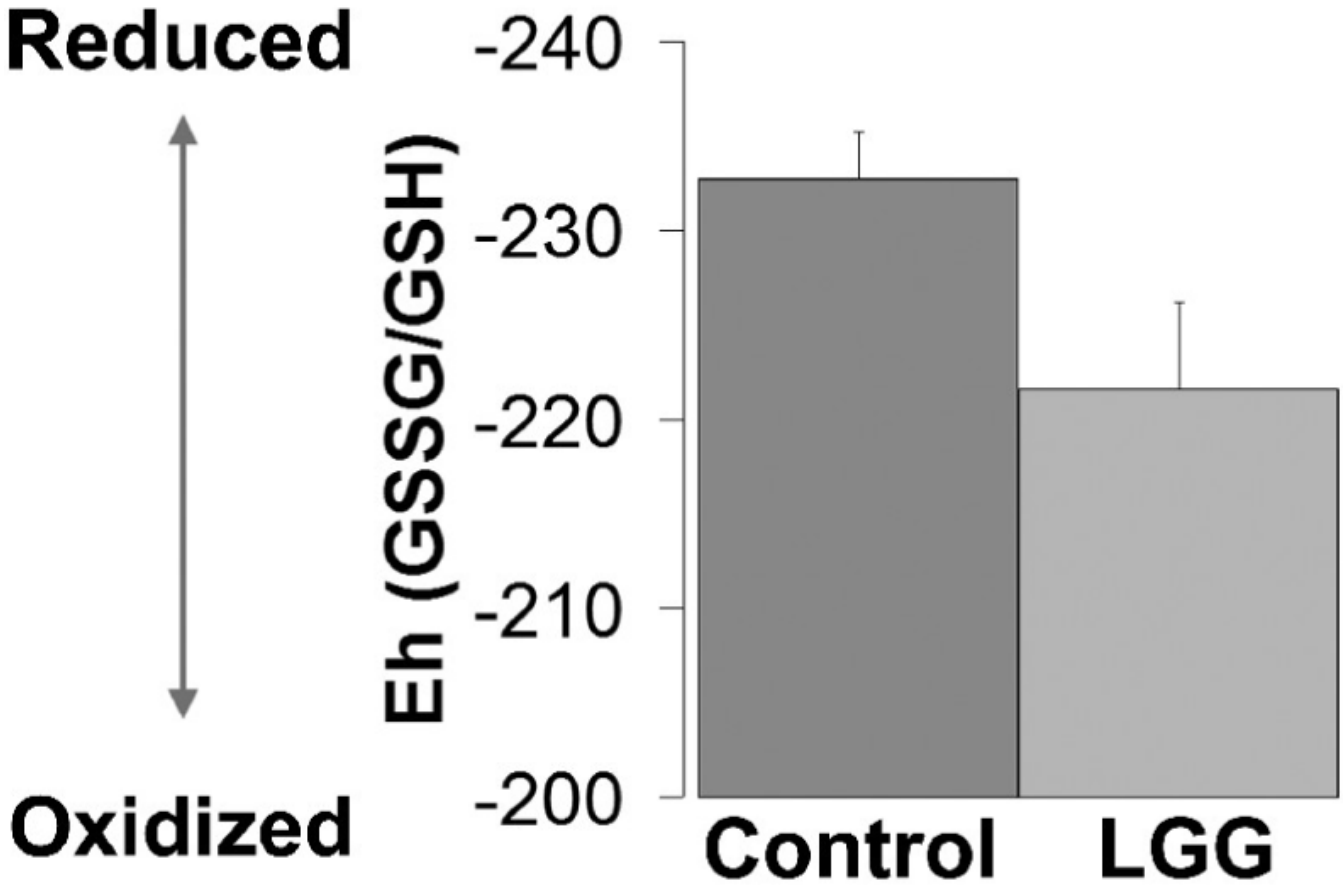

Figure 3. *LGG* induces GSH oxidation in immature intestinal epithelia

GSSG/GSH redox potential of distal small intestinal epithelia isolated from immature mice 1 hour after feeding *LGG* (light grey bar) or carrier control (dark grey bar). *$P < 0.05$ when compared to control condition.

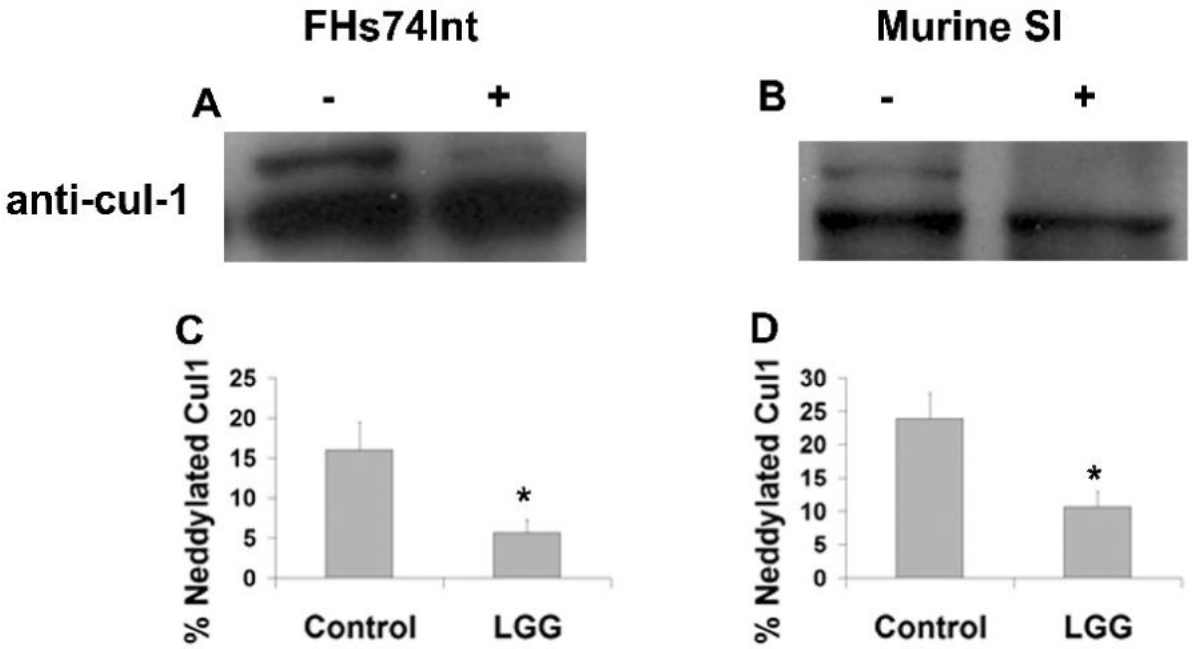

Figure 4. ***LGG*** **induces cullin-1 deneddylation in immature intestinal epithelia**

A-B. Cul1 Western blot of confluent FHs74Int cells *(A)* or of epithelia isolated from immature preweaned distal small intestines *(B)*. FHs74Int cells were collected 30 minutes after treatment *LGG* or carrier control. Small intestinal epithelia were isolated 1 hour after gavage feeding with *LGG* or carrier control. *C-D*, Quantification of percent neddylated Cul1 as determined by band density analysis of 3 representative Western blots. Data are represented as means ± SEM.

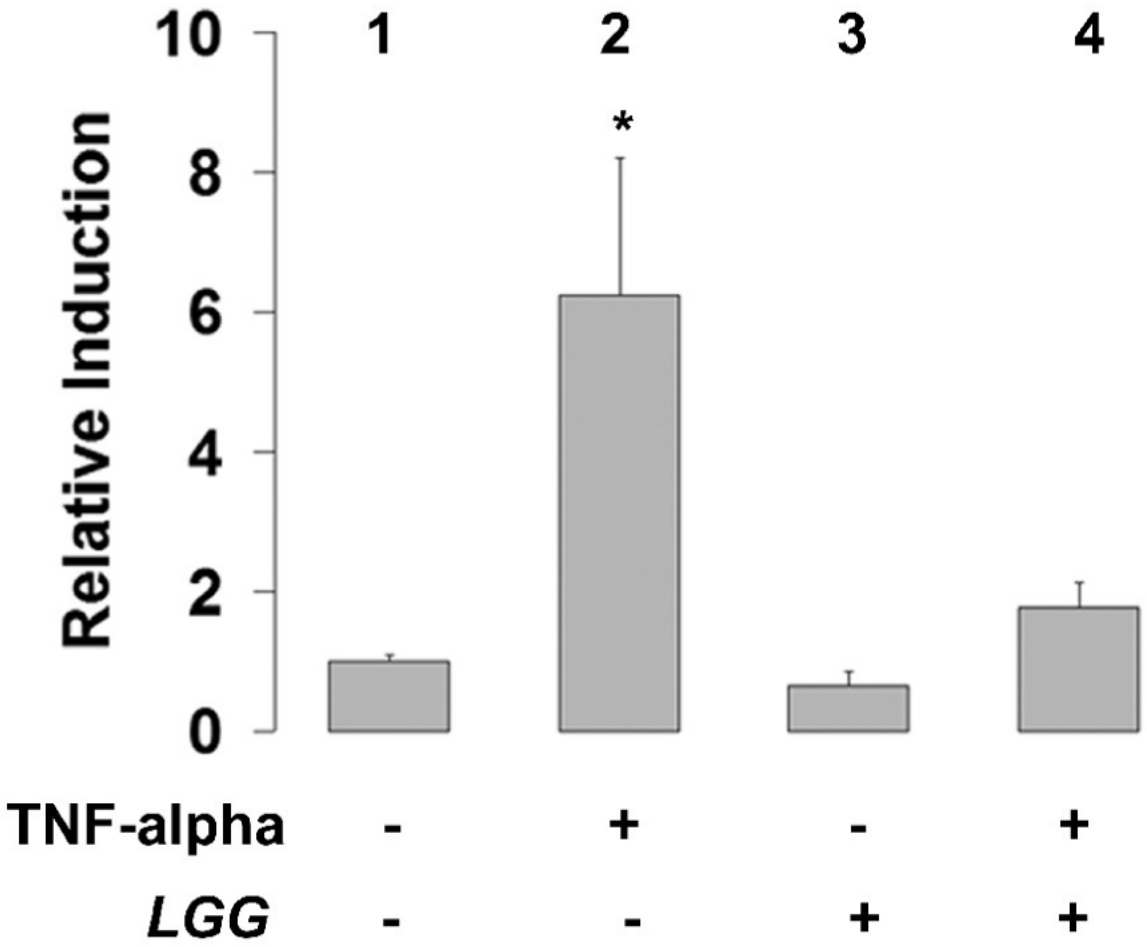

Figure 5. *LGG* prevents TNF-α-induced NF-κB activation in immature intestines

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