



Published in final edited form as:

Pediatr Res. 2008 November ; 64(5): 511–516. doi:10.1203/PDR.0b013e3181827c0f.

The Probiotic *Lactobacillus GG* May Augment Intestinal Host Defense by Regulating Apoptosis and Promoting Cytoprotective Responses in the Developing Murine Gut

Patricia W. Lin, Tala R. Nasr, Andrew J. Berardinelli, Amrita Kumar, and Andrew S. Neish

Department of Pediatrics [P.W.L., T.R.N.], Department of Pathology and Laboratory Medicine [A.J.B., A.K., A.S.N.], Emory University School of Medicine, Atlanta, GA 30322

Abstract

Necrotizing enterocolitis (NEC) remains a leading cause of morbidity and mortality in preterm infants. Although its pathogenesis is poorly understood, inappropriate apoptosis of the mucosal epithelia has been implicated. Recent clinical trials have shown probiotics may reduce the incidence of NEC, and probiotics have been shown to suppress intestinal epithelial apoptosis in cultured cells. However, little is known about their mechanism of action in the developing intestine *in vivo*. Here, we confirm that the probiotic *Lactobacillus rhamnosus GG* (*LGG*) reduces chemically induced intestinal epithelial apoptosis *in vitro*. Furthermore, we report for the first time that *LGG* administered orally to live animals can reduce chemically induced epithelial apoptosis *ex vivo*, as measured by staining for active caspase 3 and TUNEL. Utilizing cDNA microarray analysis from the intestine of live, orally inoculated mice, we show that *LGG* upregulates a battery of genes with known and likely cytoprotective effects. These studies indicate that probiotics such as *LGG* may augment intestinal host defenses in the developing intestine by stimulating anti-apoptotic and cytoprotective responses. Since apoptosis may be a precursor to NEC, understanding the mechanism behind probiotic modulation of apoptotic pathways may allow for development of more specifically targeted therapies or preventive strategies in the future.

Keywords

Necrotizing enterocolitis; probiotics; *Lactobacillus*; apoptosis; intestinal epithelia

Necrotizing enterocolitis (NEC) causes significant neonatal morbidity and mortality in very low birthweight (VLBW) infants (1). Despite advances in the supportive care of premature infants, little progress has been made in the prevention or treatment of NEC. Its etiology has not been fully elucidated, but is likely multifactorial, involving immaturity of intestinal host defenses and abnormal bacterial colonization (1-4). Recently, aberrant or excessive apoptosis has been increasingly recognized as either an initiating event or necessary step in the pathogenesis of NEC (1,5-7). Cells commonly undergo apoptosis in response to injurious stimuli (microbial, hypoxic, or chemical) (8), allowing dismantling of damaged cells without release of cellular contents and aggravation of tissue injury. However, excessive or inappropriate apoptosis may cause tissue injury and clinical consequences. Histopathologic evaluation of resected specimens from infants with surgical NEC reveal apoptosis as early

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Corresponding Author: Patricia W. Lin, M.D. 2015 Uppergate Drive Emory University School of Medicine Atlanta, GA 30322 Tel: 404-727-3360 FAX: 404-727-8538 Email: E-mail: Patricia_Lin@oz.ped.emory.edu.
Both P.W.L. and T.R.N. contributed equally to these studies.

events in the disease process (9,10), and animal models of NEC show that epithelial apoptosis precedes the gross bowel necrosis characteristic of the disease (5). Furthermore, inhibiting apoptotic pathways reduces the development of experimental NEC in rats, implying that apoptosis plays an early and important role in the pathogenesis of NEC, and modulation of this process could be exploited for therapeutic benefit (5-7).

VLBW infants at greatest risk for developing NEC are also at greatest risk of developing abnormal intestinal bacterial colonization due to exposure to nosocomial flora and frequent antibiotic administration (11). In healthy term breastfed neonates, commensal bacteria such as *Bifidobacteria* and other facultative anaerobes colonize the stool after the first 2 weeks of life (12). In contrast, VLBW and hospitalized infants exhibit delayed *Bifidobacterium* stool colonization and tend to colonize with a predominance of coliforms, other gram negative organisms, and *Streptococcus* (12). Abnormal bacterial colonization of the upper gastrointestinal tract with *Enterobacteriaceae* (13) has been reported in VLBW infants and early stool colonization with *Clostridium perfringens* has been associated with later development of NEC (14). Thus, inappropriate bacterial colonization may result in a “dysbiotic” intestinal flora that may inflict or contribute to injury of the immature gut and potentially predispose to NEC.

Probiotics are defined as ‘living micro-organisms, which upon ingestion in sufficient numbers, exert health benefits beyond basic nutrition’ (15). Probiotics can improve intestinal host defenses not only by normalizing intestinal colonization patterns but also by directly affecting intestinal epithelial function. Studies have shown commensal bacteria regulate many intestinal defenses including barrier function, mucin and IgA secretion, inflammation, and homeostatic processes such as proliferation and apoptosis (16-20). In animal models, probiotics can reduce the severity (21) and incidence (22,23) of experimental NEC. Probiotics may be effective clinically in the prevention of NEC, and bacteria studied in clinical trials include *Lactobacillus*, *Bifidobacterium*, and *Streptococcus thermophilus* (24-27). Recent *in vitro* studies indicate that the probiotic *Lactobacillus rhamnosus GG (LGG)* may be particularly effective in preventing cytokine-induced apoptosis in adult intestinal epithelial cells (20,28).

Little is known about the effect of probiotics on inducible apoptosis in immature intestines. Cytokine-mediated apoptosis occurs via the extrinsic pathway and is stimulated by ligand/death receptor interactions. As the apoptosis observed in NEC may involve physical stresses (hypoxia) and exogenous signals (bacteria, food antigens), we sought to determine if *LGG* could suppress apoptosis stimulated by multiple pathways using the broad-spectrum pro-apoptotic agent staurosporine (STS). STS has been implicated to induce both caspase-dependent (intrinsic and extrinsic) and caspase-independent apoptotic pathways through both protein kinase C (PKC) dependent and independent mechanisms (29,30). Here we report that the probiotic *LGG* reduces chemically induced (1µg/ml STS) intestinal epithelial apoptosis *in vitro*. Furthermore, we demonstrate that *LGG* decreases chemically induced apoptosis in the developing murine intestine. We modeled immature intestinal epithelia utilizing *ex vivo* organ culture of two week-old murine small intestines in which intestinal epithelial maturity resembles that of 24–28 week premature infants (31). Chemically induced intestinal epithelial cleaved caspase 3 and TUNEL staining was significantly reduced in mice prefed *LGG* as compared to carrier alone. Although both pathogenic and commensal bacteria can modulate gene regulatory responses in intestinal epithelia, we show that unlike the pathogenic bacterium *Salmonella typhimurium*, which induces genes primarily regulating proinflammatory responses(32), the probiotic *LGG*, induces genes primarily regulating cytoprotective responses in developing murine small intestines. These studies indicate that the probiotic *LGG* may exert beneficial effects on immature intestines in part by promoting anti-apoptotic and cytoprotective responses. Since apoptosis may be a precursor to NEC, understanding the mechanism behind

probiotic modulation of apoptotic pathways may allow for development of more specifically targeted therapies or preventive strategies in the future.

MATERIALS AND METHODS

Cell and bacterial culture

Rat intestinal epithelial cells (IEC-6 from ATCC) were grown to confluence on cover slips and in culture plates as previously described(8). *LGG* (provided by ATCC) was prepared overnight in *Lactobacillus* broth at 37°C per ATCC guidelines. *LGG* cultures were washed, concentrated in DMEM, and applied to IEC-6 cells at 10⁷ CFUs/ml or gavage fed to 2 week-old mice at 10¹⁰ CFUs/ml.

In vitro experiments

IEC-6 cells were pretreated in media with or without *LGG* for 2 hours in a 5% CO₂ incubator at 37°C. Apoptosis was then induced using STS (1µg/ml) or DMSO control for 2 hours. Numbers of TUNEL positive nuclei were counted per high power field (HPF).

Animal care

All animal experiments were approved by the Institutional Animal Care and Use Committee at Emory University. C57BL/6J mice were bred in an animal facility at Emory University. 2 week-old mice were orally gavage fed 0.2ml of DMEM with or without *LGG*. After 4 hours, the mice were anesthetized in CO₂ and then euthanized by cervical dislocation. Small intestines were isolated and immediately frozen in TRIzol (Invitrogen) for RNA isolation, fixed in 10% formalin for histologic staining, or utilized in *ex vivo* experiments.

Ex-vivo experiment

Intestines were surgically excised and opened lengthwise to expose the intestinal epithelia. *Ex vivo* intestines were then maintained in RPMI media in 24-well cell culture plates at 37°C and apoptosis induced with STS (1µg/ml) for 2 hours. Intestines were subsequently washed in PBS and immediately fixed in 10% formalin for histologic staining.

Histologic staining

After experimental treatment, cells on coverslips were washed and fixed in 4% Paraformaldehyde (Fischer) or intestinal tissue was fixed, paraffin embedded, and sections mounted on slides. *TUNEL*: Apoptotic cells were labeled by an InSitu Cell Death Detection Kit (Roche), using terminal deoxynucleotidyltransferase (TUNEL) according to manufacturer guidelines. *In vitro counterstaining*: Cells on coverslips were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1µg/ml). *Cleaved caspase 3*: Cells containing activated caspase 3 were detected using cleaved caspase 3 (Asp 175) antibody for immunohistochemistry (Cell Signaling) according to manufacturer's guidelines. Slides were treated with primary cleaved caspase 3 antibody overnight (1:50) followed by secondary antibody 1:500 for 1 hour (HRP-conjugated anti-rabbit IgG, Amersham Biosciences). Sigma Fast DAB tablet set (Sigma-Aldrich) was used as per manufacturers instructions. *Ex vivo counterstaining*: Slides were counterstained with hematoxylin. *Quantification and statistical analysis*: Number of positive cells was counted in a minimum of 10 HPFs. Statistical differences were analyzed by ANOVA.

cdNA microarray analysis

Total RNA isolated from homogenized intestinal tissue was subjected to DNase I digestion, purified (Qiagen kit), and quality verified by gel electrophoresis. Total sample RNA (30 µg) and universal mouse reference RNA (20 µg) was used to synthesize cDNA labeled with fluorlink Cy5 dCTP and Cy3 dCTP, respectively. Sample and reference cDNA were

cohybridized onto the MEEBO mouse 38.5K gene chip (Vanderbilt MicroArray Shared Resources, Vanderbilt University). Washed and dried chips were scanned (GenePix 4100A), and images were captured and analyzed (GenePix Pro 5.0). Ratio of Cy5: Cy3 of untreated and treated samples was calculated to assess relative changes in gene expression. Fold induction of greater than 2.5 was considered positive.

Real-time quantitative reverse transcription-PCR (RT-PCR)

Total RNA was reverse transcribed from random hexamer primers using Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA). Real-time quantitative RT-PCR analysis (SYBR Green real-time PCR assay, Applied Biosystems) was performed on the reverse transcription cDNA products using primers for *Dusp3*, *Areg*, *CDK2*, *Xrcc6*, *FoxF2*, *Errb2*, *Sox4*, and *Jak2* (Primer Express software, Applied Biosystems) and 18s rRNA (Taqman rRNA control reagents kit, Applied Biosystems) as described previously(33). Level of expression for a given gene was normalized to the 18s rRNA level of the same sample. Fold difference was the ratio of the normalized value of each experimental sample to that of control samples. Primer sequences are available upon request.

RESULTS

LGG reduces chemically- induced intestinal epithelial apoptosis *in vitro*

LGG has been shown to suppress cytokine-induced apoptosis *in vitro* in cultured epithelial cells (20). As mentioned previously, intestinal epithelial apoptosis observed to precede the gross histologic changes of NEC might be caused by multiple pathways not represented when using cytokines as a stimulant. Thus, to determine whether *LGG* could suppress apoptosis stimulated by multiple pathways, we used two intestinal epithelial cell lines, IEC-6 (rat) and Caco-2 (human) and the broad-spectrum pro-apoptotic agent, STS. Apoptosis was assayed by TUNEL staining. As expected, STS induced increased TUNEL staining in IEC-6 cells compared to media control (Fig. 1A, compare panels 1 and 2; and 1B, bars 1 and 2). Further, while *LGG* alone had no measurable effect on intestinal epithelial apoptosis, *LGG* pretreatment reduced STS-induced TUNEL positivity (Fig. 1A, compare panels 3 to 1; and B, bars 4 to 2). Essentially identical data were obtained in Caco-2 cells (data not shown). These results confirm that *in vitro*, *LGG* can reduce intestinal epithelial apoptosis stimulated with a broad-spectrum pro-apoptotic agent in both rodent and human cell lines.

LGG decreases chemically induced epithelial apoptosis in the developing murine gut

To determine whether *LGG* has comparable effects on intestinal epithelial apoptosis in the developing gut, we modeled the developing intestine in two week-old preweaned mice (epithelial maturity of 24–28 premature human intestine) (31) and tested intestinal epithelial sensitivity to apoptosis *ex vivo*. We fed these immature mice media with or without *LGG* by oral gavage. Four hours later we surgically excised the small intestines for *ex vivo* chemical induction of apoptosis. Previous intestinal transit time experiments with aniline dye demonstrated that orally gavaged material reaches the large intestine by 1 hour, and culture studies confirmed recovery of 1000-fold more live *Lactobacillus* in small intestines from *LGG*-fed mice at 4 hours post-gavage. As expected, control intestines isolated from mice fed media alone and stimulated *ex vivo* with STS demonstrated significantly increased epithelial TUNEL staining (especially at villus tips) compared to untreated intestines (Fig. 2A, compare panels 1 and 2; and 2B, first and second bars). Intestines harvested from mice treated with *LGG* exhibited significantly reduced epithelial apoptosis after *ex vivo* STS treatment (Fig. 2A, compare panels 2 and 4; and 2B, second and fourth bars). *LGG* alone had no effect (Fig. 2A, panels 1 and 3; and 2B, first and third bars). Next, we performed cleaved caspase 3 immunohistochemistry on the harvested intestines to test whether *LGG* reduces STS-induced apoptosis by inhibiting caspase 3 activation (final executioner caspase activity required for

both intrinsic and extrinsic caspase-dependent apoptosis). As predicted, increased activated caspase 3 staining occurred after *ex vivo* STS treatment (Fig. 3A, panels 1 and 2; and 3B, first and second columns). Animals orally fed *LGG* before *ex vivo* experimental exposure exhibited significantly less caspase 3 activation as compared to those fed media alone (Fig. 3A, panels 2 and 4; and 3B, second and fourth columns). These studies indicate that *LGG* can reduce chemically induced apoptosis and caspase 3 activation in the developing murine intestine.

LGG upregulates cytoprotective gene expression in the developing murine intestine

To further investigate the possible mechanism behind *LGG* reduction of experimental apoptosis in the immature intestine, we performed cDNA microarray analysis on intestines from our 2 week-old model of the developing murine gut. We collected small intestines from immature mice 4 hours after feeding *LGG* versus media alone. Based on previous intestinal transit time experiments, we expected that small intestinal epithelia would be exposed to the probiotic bacteria for 2–3 hours before harvesting. We have previously demonstrated that pathogenic bacteria such as *Salmonella typhimurium* can upregulate proinflammatory responses which in turn induce anti-apoptotic gene transcription (8). Here, we have analyzed the genes upregulated after oral feeding with *LGG* (Table 1, fold induction comparing bacteria fed to media fed mice). *LGG* induced upregulation of 185 genes and downregulation of 2400 genes in the small intestine of immature mice. 33 of the upregulated genes regulate putative cytoprotective processes (Table 1). In contrast to the primarily proinflammatory gene transcription pattern found after oral gavage of *Salmonella typhimurium*, genes upregulated after feeding *LGG* primarily regulate homeostatic processes such as cellular proliferation and migration and mitogen activated protein kinase (MAPK) pathways known to be important for growth, differentiation, and cytoprotection. Furthermore, unlike *Salmonella typhimurium* which induced anti-apoptotic gene upregulation through NF- κ B and proinflammatory activation(8), *LGG* induced anti-apoptotic gene transcription without upregulation of proinflammatory genes. Selected genes (Table 1, italicized) upregulated by *LGG* as measured by cDNA microarray analysis were confirmed by quantitative real-time RT-PCR (Fig. 4). These results suggest that *LGG* may prevent apoptotic responses by upregulating anti-apoptotic and cytoprotective pathways in the developing gut.

DISCUSSION

Probiotics have been shown to reduce the severity and incidence of both experimental NEC in animals (21-23) and clinical NEC in some small single center trials (24-27). Although these studies have stimulated increased interest worldwide in the potential use of probiotics to prevent NEC in VLBW infants, little work has been done to understand the mechanism by which probiotics exert a beneficial effect on the immature intestine (34). We show in this study that *LGG*, a well-studied probiotic bacterium that is also a member of the commensal flora, may protect immature intestines from apoptotic stimuli by upregulating a panel of anti-apoptotic/cytoprotective genes. Apoptosis is a form of programmed cell death that allows elimination of irreversibly damaged cells while preserving overall tissue function, and plays a vital role in normal developmental processes. However, overwhelming activation of apoptosis may result in tissue failure. Epithelial apoptosis has been described in the early stages of animal models of NEC where rat pups were subjected to repeated episodes of hypoxia, formula feeding and cold stress (5). Clinically, early NEC may also be due pro-apoptotic stresses in the gut due to hypoxia secondary to hypoperfusion, chemical injury from digestive products, or direct pro-apoptotic signaling from microbial stimuli.

In health, apoptosis is kept in check by basal expression of a spectrum of anti-apoptotic molecules such as the IAPs and anti-apoptotic members of the Bcl family that serve to inhibit the molecular apoptotic machinery. Additionally, these effectors are typically rapidly induced

during inflammatory responses to counter increased exogenous stresses that accompany inflammation. Thus, transcriptional upregulation of anti-apoptotic effectors is considered characteristic of inflammatory signaling. It is very well-established by our laboratory and others that enteric pathogens elicit marked changes in epithelial gene regulation within 1–2 hours of exposure (32,35–37), and similar experiments indicate that the adult gut can also respond to colonization with commensals (16,32), though clearly the expression profiles are highly different. Our array data shows that probiotic bacteria are capable of stimulating new gene activation in the immature gut that is measurably different to that induced by classical enteric pathogens. These genes include not only known anti-apoptotic genes (Ku 70 (38), Sox4 (39), Phip (40), Api5 (41), Jak2 (42)), but also candidate anti-apoptotic genes which regulate MAPK pathways (Dusp3 (43)). Multiple genes regulating cellular proliferation and migration as well as genes regulating MAPK pathways implicated in growth and development were also upregulated (Table 1). These genes may be cytoprotective by aiding in intestinal growth and healing, which may be especially important homeostatic processes in the developing gut.

How non-pathogenic bacteria activate epithelial signaling pathways is a topic of intense investigation. Classically, pathogens are monitored by cellular pattern recognition receptors such as the toll-like receptors (TLRs). Commensals also express TLR ligands such as LPS, PGN and flagellin, but in the context of intact barrier function, do not cause overt cellular inflammation. TLR ligands and other bacterial products clearly affect epithelial signaling, gene transcription and mediate cytoprotective effects, and TLR signaling is clearly necessary for the overall integrity of the mucosa (8,44–46). Likely, the healthy gut requires a sub-inflammatory threshold of stimulation by bacteria or their products (TLR ligands, formylated peptide metabolic products such as butyrate) to maintain health. This concept of an optimal healthy bacterial ecosystem can be contrasted with a putative “dysbiotic” flora that can be associated with inflammatory disease (47). This is consistent with the observation that certain strains of bacteria colonized into the immature gut are associated with NEC predisposition (13,14). In this light, *Lactobacillus* and other probiotics could plausibly exert beneficial effects by correcting a “dysbiotic” flora, providing predictable and beneficial exposure to bacterial factors, and consequently inducing constitutive stimulation of cytoprotective genes.

Since probiotic therapy in VLBW infants requires administration of live bacteria to patients with immature immunity, its use should not be taken lightly. While not yet reported in VLBW infants, probiotics can cause invasive disease in immunocompromised populations (48). Prebiotics (nondigestible dietary supplements which promote growth of commensal bacteria) and post-biotics (bacterial metabolites such as butyrate) can mediate beneficial effects on intestinal health. Certain *LGG* proteins have been shown to prevent cytokine-induced (28) and STS-induced apoptosis (data not shown) *in vitro*. However, our studies indicate that these bacterial products may not be as effective when administered orally to immature mice *in vivo* (data not shown). Future studies, which aim to better understand the beneficial actions of probiotics, without use of live microorganisms, are needed in order to develop safer alternative therapies in our patients.

Finally, the ability of *LGG* to induce anti-apoptotic and cytoprotective pathways without inflammation further underscores the importance of promoting growth of commensal bacteria in the developing intestine. Since apoptosis is an important precursor in the pathogenesis of NEC (5,9,10) and its resultant exuberant inflammatory response (septic shock, a major cause of morbidity and mortality), understanding how to inhibit apoptosis without inducing inflammation could prove invaluable in developing future preventive strategies for this devastating disease.

Acknowledgments

Financial Support: This work was supported by an Emory Eggleston Children's Research Center seed grant (P.W.L.), the National Institutes of Health grants DK062851 and DK076613 (P.W.L.), AI051282 and DK071604 (A.S.N.), and Emory Digestive Disease Research Center Grant R24 DK-064399.

Abbreviations

HPF, high power field; LGG, *Lactobacillus rhamnosus* GG; STS, Staurosporine; VLBW, very low birthweight.

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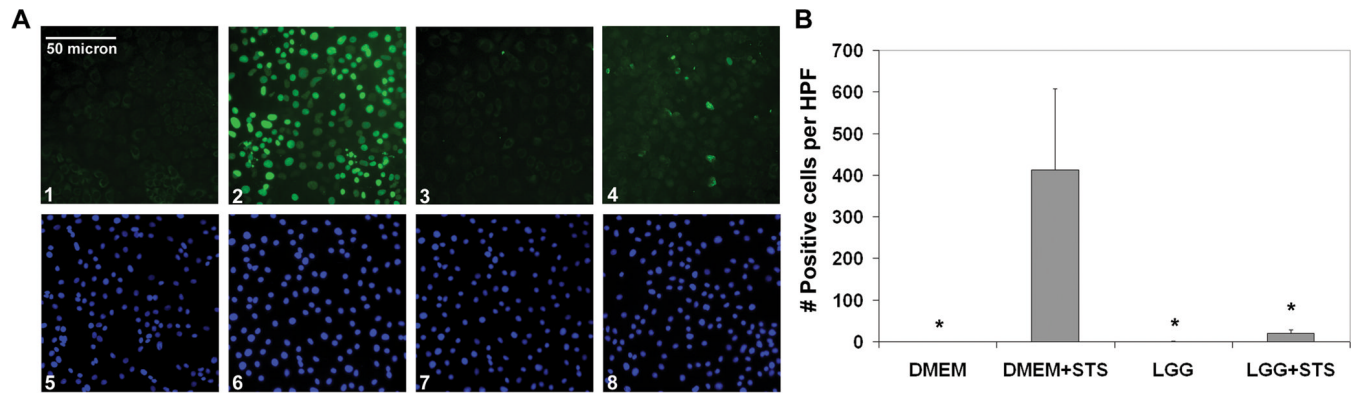


Figure 1. LGG reduces chemically induced apoptosis in IEC-6 cells

A, (1–4), TUNEL staining of IEC-6 cells treated with STS after preincubation with DMEM with (4) or without (2) LGG or treated with carrier control after preincubation with DMEM with (3) or without (1) LGG. (5–8), DAPI nuclear counterstaining of identical IEC-6 cells.

B, Average number (mean \pm SD) of TUNEL positive IEC-6 cells per HPF per treatment condition as indicated. Representative of 3 independent experiments. * $P < 0.05$ as compared to DMEM prefed, STS stimulated intestines (bar 2).

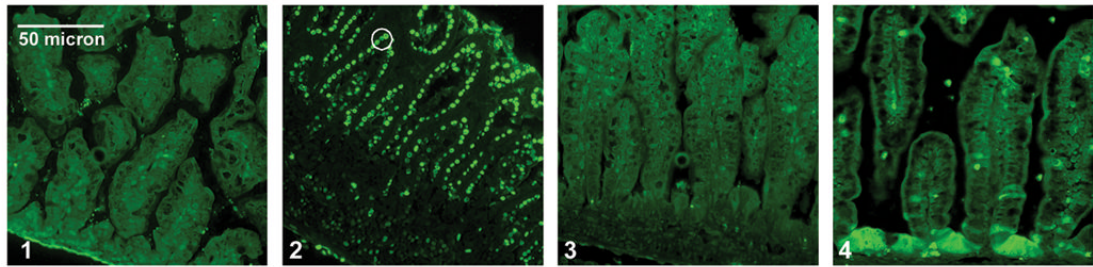
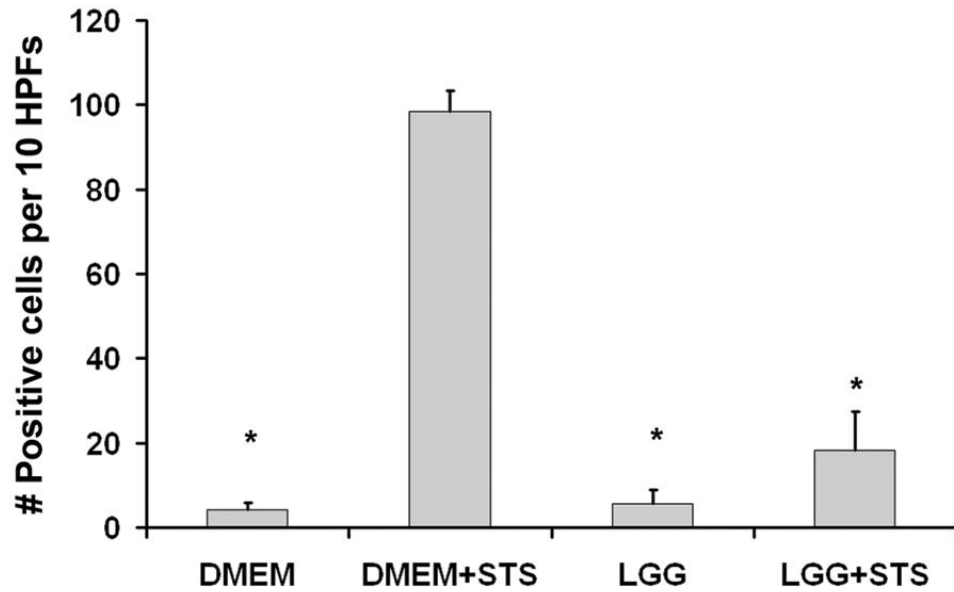
A**B**

Figure 2. *LGG* reduces chemically induced intestinal epithelial apoptosis in the developing murine gut

A, TUNEL staining of small intestines collected from 2 week-old mice prefed DMEM with (3) or without (1) *LGG* alone, or stimulated with STS *ex vivo* after prefeeding DMEM with (4) or without (2) *LGG*. Apoptotic cells noted by fluorescent green nuclei such as those circled (2). **B**, Average number (mean \pm SD) of TUNEL positive intestinal epithelial cells per 10 HPFs per treatment condition as indicated ($n=3$ experiments). * $P < 0.05$ as compared to DMEM prefed, STS stimulated intestines (bar 2).

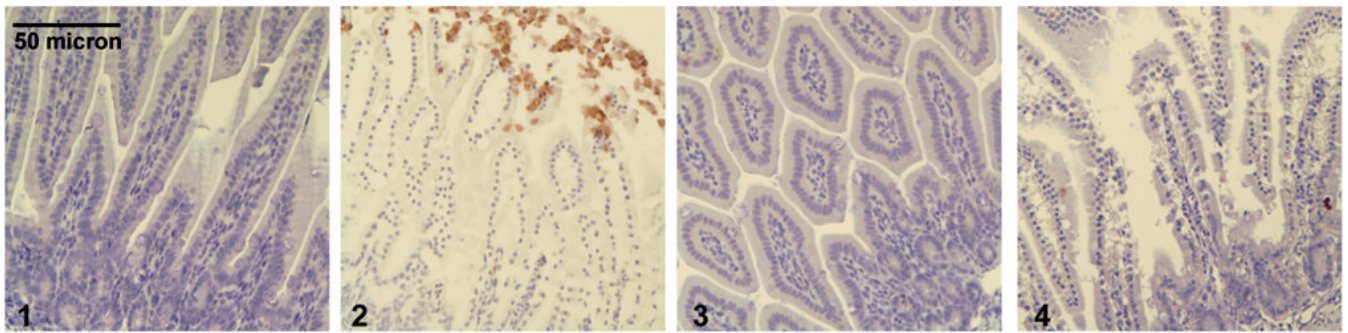
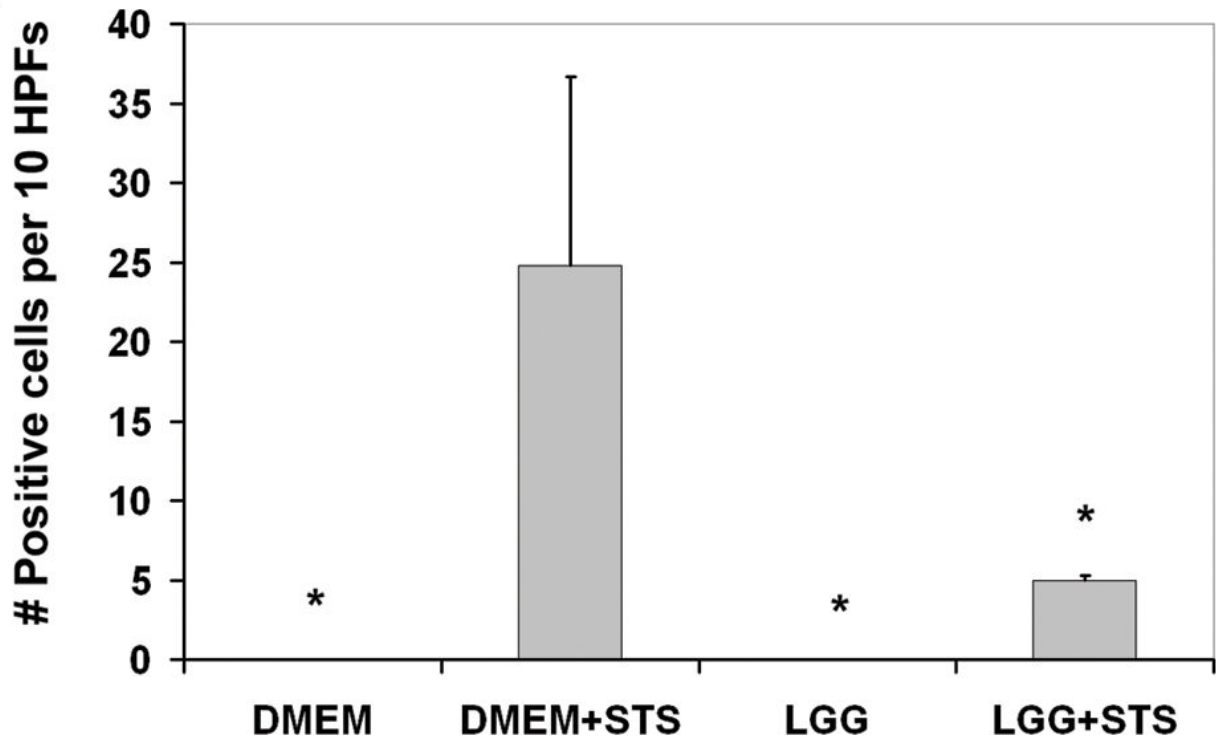
A**B**

Figure 3. *LGG* reduces chemically induced intestinal epithelial caspase 3 cleavage in the developing murine gut

A, Cleaved caspase 3 staining of small intestines collected from 2 week-old mice prefed DMEM with (3) or without (1) *LGG* alone, or stimulated with STS *ex vivo* after prefeeding DMEM with (4) or without (2) *LGG*. **B**, Average number (mean \pm SD) of TUNEL positive intestinal epithelial cells per 10 HPFs per treatment condition as indicated ($n=3$ experiments). * $P < 0.05$ as compared to DMEM prefed, STS stimulated intestines (bar 2).

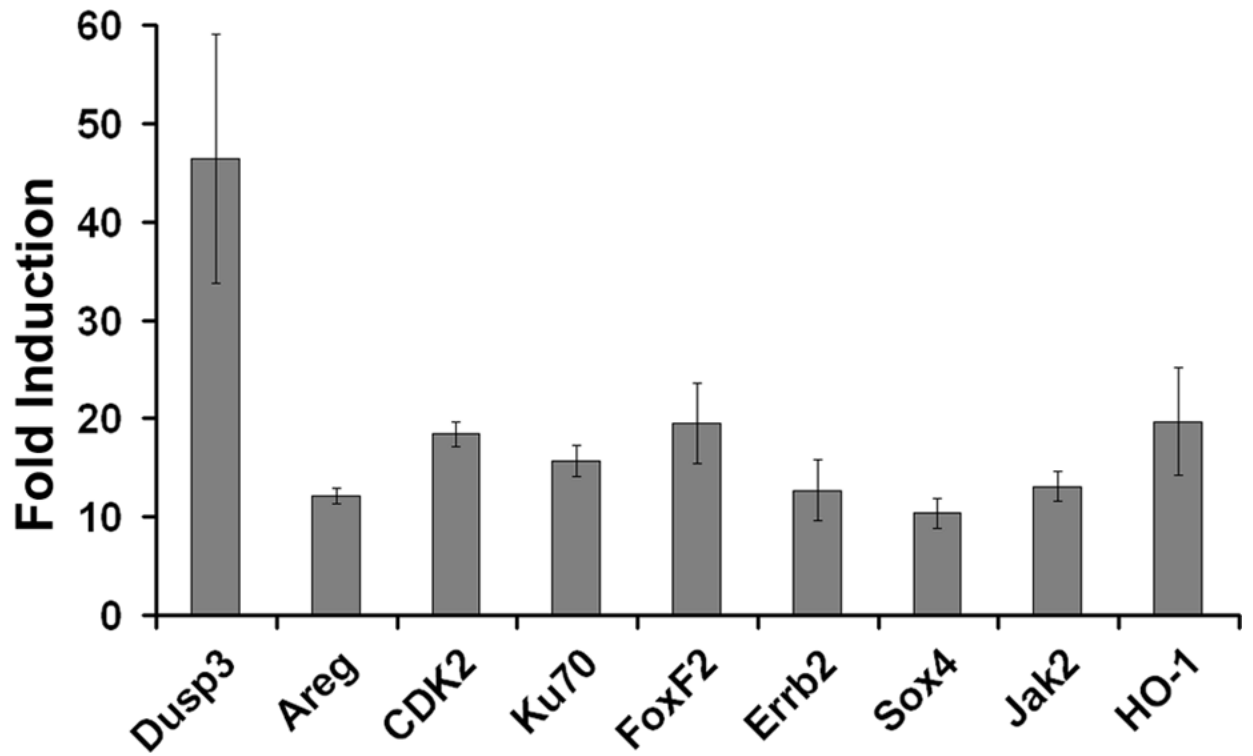


Figure 4. *LGG* upregulates mRNA expression of anti-apoptotic and cytoprotective genes
Quantitative real-time RT-PCR of genes expressed in the small intestine of *LGG* vs DMEM fed 2 week-old preweaned mice. Bars represent average fold induction (mean \pm SD) when comparing mRNA levels in *LGG* to DMEM fed mice ($n=3$ experiments).

Table 1
***LGG* upregulates anti-apoptotic and cytoprotective genes by cDNA microarray**
 Selected upregulated genes in small intestines of *LGG* vs DMEM fed 2 week-old mice by cDNA microarray. Numbers represent average fold induction when comparing *LGG* to DMEM fed mice ($n=3$).

CATEGORY	NAME	FOLD INDUCTION
Cell Proliferation/Migration		
	Guanine nucleotide binding protein, alpha q (Gnaq)	26.1
	LIM and senescent like antigen domains (Lims1/PINCH)	7.2
	Tight junction protein 2 (Tjp2/ZO-2)	5.9
	Carbonic anhydrase 8 (Car8)	5.7
	Frizzled homolog 1 (Fzd1/Fz-1)	5.0
	<i>Amphiregulin (Areg)</i>	4.6
	<i>Cyclin-dependent kinase 2 (Cdk2)</i>	4.5
	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D (Sema6D)	4.4
	Adenomatosis polyposis coli (APC)	4.0
	Choline phosphotransferase 1 (Chpt1)	4.0
	Homeodomain interacting protein kinase 1 (Hipk1)	3.7
	A kinase (PRKA) anchor protein (yotiao) 9	3.5
	Ephrin B2 (Efnb2)	3.5
	Proliferating cell nuclear antigen (Pcna)	3.4
	<i>Forkhead box F2 (FoxF2)</i>	3.4
	Integrin beta 1 binding protein 1 (Itgb1bp1/Icap-1)	3.3
	Calpain 7 (clpn7)	3.3
	Microtubule-associated protein, RP/EB family, member 3	2.8
	Zinc finger protein 326	2.8
	Cell division cycle associated 7 (Cdca7)	2.6
	Kinase insert domain protein receptor (Kdr)	2.6
MAP Kinase Related		
	<i>Dual Specificity Phosphatase 3 (Dusp3/VHR)</i>	53.2
	Guanine nucleotide binding protein, alpha inhibiting (Gnai3)	13.7
	Protein tyrosine phosphatase receptor, type E (Ptpre)	4.8
	<i>ErbB2 interacting protein (ErbB2ip/Erbin)</i>	3.2
	Neural precursor cell expressed, developmentally down-regulated gene 4-like (Nedd4l/Nedd4b)	2.6
	CREB/ATF bZIP transcription factor	2.6
Anti-Apoptotic		
	Xray repair complementing defective repair (Xrcc6/Ku70)	4.2
	Zinc finger CCCH-type containing 15 (Zc3h15)/ Erythropoietin 4 immediate early response	4.0
	<i>SRY-box containing gene 4 (Sox4)</i>	3.1
	Pleckstrin homology domain interacting protein (PHIP)	3.0
	Apoptosis inhibitor 5 (Api5/AAC-11)	2.8

CATEGORY	NAME	FOLD INDUCTION
	<i>Janus kinase 2 (Jak2)</i>	2.7