Probiotic *Lactobacillus rhamnosus* Inhibits the Formation of Neutrophil Extracellular Traps

Linda Vong, Robert J. Lorentz, Amit Assa, Michael Glogauer and Philip M. Sherman

*J Immunol* published online 24 January 2014
http://www.jimmunol.org/content/early/2014/01/24/jimmunol.1302286

Supplementary Material

http://www.jimmunol.org/content/suppl/2014/01/24/jimmunol.1302286.DCSupplemental

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Probiotic *Lactobacillus rhamnosus* Inhibits the Formation of Neutrophil Extracellular Traps

Linda Vong,* Robert J. Lorentz,* Amit Assa,† Michael Glogauer, † and Philip M. Sherman*†

Neutrophil extracellular traps (NETs) are an essential component of the antimicrobial repertoire and represent an effective means by which neutrophils capture, contain, and kill microorganisms. However, the uncontrolled or excessive liberation of NETs also damages surrounding cells and can contribute to disease pathophysiology. Alterations in the gut microbiota, as well as the presence of local and systemic markers of inflammation, are strongly associated with the manifestation of a spectrum of intestinal disorders, including chronic inflammatory bowel disease. Although probiotics exert beneficial effects on gut homeostasis, their direct effect on neutrophils, which are abundant in the setting of intestinal inflammation, remains unclear. In this study, we investigated the effects of nonpathogenic, enteropathogenic, and probiotic bacteria on the dynamics of NET formation. Using murine bone marrow–derived neutrophils and the neutrophil-differentiated human myeloid cell line d.HL-60, we demonstrate for the first time, to our knowledge, that probiotic *Lactobacillus rhamnosus* strain GG inhibits both PMA- and *Staphylococcus aureus*–induced formation of NETs. Moreover, probiotic *L. rhamnosus* strain GG had potent antioxidative activity: dampening reactive oxygen species production and phagocytic capacity of the neutrophils while protecting against cell cytotoxicity. Within the milieu of the gut, this represents a novel mechanism by which probiotics can locally dampen innate immune responses and confer desensitization toward luminal Ags. *The Journal of Immunology*, 2014, 192: 000–000.

Neutrophils are one of the first cells recruited to sites of microbial challenge and are elegantly equipped with an array of antimicrobial and proteolytic enzymes, reactive oxygen species (ROS), and anti-inflammatory molecules to combat infection (1, 2). The recent discovery of neutrophil extracellular traps (NETs) (3) has expanded the view of how neutrophils capture, contain, and kill microorganisms. Brinkmann et al. (3) first described that, following either microbial or pharmacologic activation, neutrophils release web-like extracellular structures (composed primarily of decondensed chromatin) that are highly decorated with antimicrobial and granule proteases. In a given niche, the formation of NETs, termed NETosis, is sufficient to trap bacteria (3, 4) and prevent further dissemination (5, 6). Moreover, its importance is underscored by evidence of persistent and recurrent infections in individuals with neutrophils that are not able to form NETs (7, 8). However, there is emerging evidence suggesting that uncontrolled or excessive NETosis, and the associated liberation of cell-free DNA and degradative proteases, damages surrounding cells and contributes to disease pathophysiology (9–11).

The intestinal tract serves as a unique interface between cells of the immune system and the Ag-rich, luminal environment. A delicate balancing act is maintained to ensure that robust host inflammatory responses are mounted against invasive pathogenic microorganisms and yet immune tolerance conferred to commensal inhabitants (12). Disturbance of the gut microbiota, termed “dysbiosis,” results in pathophysiological consequences (13).

Supplementation with beneficial microbes (also referred to as probiotics) can delay or prevent the onset of experimental colitis and, in humans, induce and maintain remission in individuals with ulcerative colitis (14, 15). In premature infants, the risk for developing necrotizing enterocolitis (aberrant inflammation of the intestine in response to initial bacterial colonization) is significantly reduced by supplementation with probiotics (16). Among their arsenal of properties, such as a strain-specific ability to induce colonization resistance, maintain intestinal epithelial integrity, increase protective goblet cell–derived mucus production, and stimulation of the host immune system, probiotics also can produce soluble antimicrobial and other modulatory factors that serve to counteract dysbiosis and protect against intestinal inflammation (17).

In the context of the intestinal tract, little is known about the ability of gut microorganisms to induce NETs. Given that neutrophils are abundant in the setting of colonic inflammation and the close proximity of these granulocytes to luminal and mucosa-associated microorganisms, this study sought to determine the effects of commensal, probiotic, and enteropathogenic bacteria on the formation of NETs. To our knowledge, we demonstrate for the first time that probiotic *Lactobacillus rhamnosus* strain GG (LGG) inhibits both pharmacologic and pathogen-induced NETosis. Moreover, neutrophils exposed to LGG have a reduced capacity to undergo oxidative burst and phagocytosis.

*Cell Biology Program, Division of Gastroenterology, Hepatology, and Nutrition, Peter Gilgan Centre for Research and Learning, Hospital for Sick Children, Toronto, Ontario M5G 0A4, Canada; and†Faculty of Dentistry, University of Toronto, Toronto, Ontario M5G 1G6, Canada Received for publication August 27, 2013. Accepted for publication December 19, 2013.

This work was supported by operating grants from the Canadian Institutes of Health Research (MOP-89894 and IOP-92890). P.M.S. is the recipient of a Canada Research Chair in Gastrointestinal Disease.

Address correspondence and reprint requests to Dr. Philip M. Sherman, Cell Biology Program, Research Institute, Hospital for Sick Children, 555 University Avenue, Room 8263, Toronto, ON M5G 1X8, Canada. E-mail address: philip.sherman@sickkids.ca

The online version of this article contains supplemental material.

Abbreviations used in this article: BMDN, bone marrow–derived neutrophil; LDH, lactate dehydrogenase; LGG, *Lactobacillus rhamnosus* strain GG; MOI, multiplicity of infection; *N.1917*, *Escherichia coli* strain Nissle 1917; NET, neutrophil extracellular trap; R0011, *Lactobacillus rhamnosus* strain R0011; ROS, reactive oxygen species.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/516.00
Materials and Methods

Ethics statement

All animal work was approved by the Hospital for Sick Children’s Animal Research Ethics Board (approval number 22577) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Bacterial strains

Probiotic LGG (ATCC 53103), and L. rhamnosus strain R0011 (R0011; Lallemand Health Solutions, Montreal, QC, Canada), and Escherichia coli strain Nissle 1917 (N1917) (18) were grown for 16 h at 37°C in static non-aerated deMan–Rogosa–Sharpe or Luria–Bertani broth (Difco Laboratories, Detroit, MI), respectively. Aliquots of bacterial cultures subsequently were used to inoculate fresh broth and then cultured for an additional 3 h prior to use. Commensal E. coli strains HB101 (serotype O:rough) (19) and F18 (serotype O:rough:K1:H5) (20), enterohemorrhagic strains EDL933 (serotype O157:H7) (18) and CL56 (serotype O157:H7) (19), prototype translocating strain C25 (serotype O:rough non-motile) (21) (Invitrogen), and adherent invasive LFR2 (serotype O8:K1:H2) (22), a generous gift from Dr. A. Darfeuille-Michaud, UMR 1071 Inserm, Université d’Auvergne, Clermont-Ferrand, France, were grown in Penassay broth (Difco Laboratories), as above. Staphylococcus aureus (ATCC 25923) was grown in tryptic soy broth (Difco Laboratories). Following centrifugation (4000 × g, 5 min) and three washes in HBSS (Invitrogen), bacterial strains were normalized to 105 CFU/ml. In some experiments, bacteria were fixed in 4% formaldehyde, washed, and resuspended in HBSS. Loss of viability was confirmed by plating formalin-fixed bacteria onto agar plates (37°C for 48 h).

Bone marrow–derived and cultured neutrophils

Murine bone marrow–derived neutrophils (BMDNs) were harvested from male C57Bl/6 mice, as described previously (23). Briefly, the tibia and femur were isolated and flushed with MEM alpha (Invitrogen), and centrifuged at 400 g. The resultant cell suspension was centrifuged at 4000 g for 10 min. The cell pellet was resuspended in PBS (pH 7.4, without Ca2+/Mg2+/EGTA; Life Technologies), layered on top of a Percoll density gradient (80/65%/55%; Sigma Aldrich), and centrifuged at 1000 g for 30 min. BMDNs at the 80/65% interface were collected and washed in PBS, and contaminating RBCs were removed by hypotonic lysis. BMDNs were resuspended in HBSS (with Ca2+/Mg2+/EGTA; Invitrogen) and normalized to 1 × 107 cells/ml.

The human promyelocytic cell line HL-60 (ATCC CCL-240) was maintained in IMDM supplemented with 20% heat-inactivated FBS (both from Invitrogen). To differentiate HL-60 cells into a mature neutrophil phenotype (d.HL-60), 2 × 106 cells/ml were incubated with 1% DMSO for 5 days and subsequently normalized to a concentration of 1 × 106 cells/ml in HBSS (with Ca2+/Mg2+/EGTA; Invitrogen) and Invitrogen). For all experiments, 1 × 106 BMDNs or d.HL-60 cells were used.

Quantification and visualization of NETs

BMDNs and d.HL-60 cells were preincubated with either live or formalin-fixed LGG (1 h, 37°C, 5% CO2) and then activated to form NETs with the phorbol ester PMA (100 nM) or LGG (1 h, 37°C, 5% CO2). Oxidation and formation of formazan, which is produced in proportion to the level of LDH in cell supernatants, were measured at 490 nm in a microplate reader (Victor X3; PerkinElmer).

ROS production

ROS and superoxide production by BMDNs and d.HL-60 cells was quantified by luminol- and lucigenin-ECL, respectively. Briefly, 105 BMDNs and d.HL-60 cells were pretreated with live or formalin-fixed LGG (MOI 1, 10, and 100; 1 h, 37°C) or with bacterial strains N1917, HB101, EDL 933, CL56, or LF28 (MOI 100) and then activated with PMA (100 nM, 1 h). To measure ROS levels, luminol (50 μM) and HRP (1.2 U/ml) were added to the reaction. In some experiments, BMDNs and d.HL-60 cells were activated with H2O2 (10 nM), and the resulting ROS levels were measured. To quantify PMA-induced superoxide production, lucigenin (5 μM) was added to the reaction mixture. The resulting chemiluminescence was measured using a microplate reader (VICTOR X3; PerkinElmer).

Cell cytotoxicity

Lactate dehydrogenase (LDH) release from PMA- or S. aureus–treated BMDNs and d.HL-60 cells was quantified using the Cytoscan LDH Cytotoxicity Kit (Bioscience). Briefly, BMDNs and d.HL-60 cells were treated with either live or formalin-fixed LGG (1 h, 37°C, 5% CO2) and then activated with PMA (100 nM) or S. aureus (107 CFU) for 1 h (37°C, 5% CO2). Oxidation and formation of formazan, which is produced in proportion to the level of LDH in cell supernatants, were measured at 490 nm in a microplate reader (VICTOR X3; PerkinElmer).

Phagocytosis assay

Phagocytic capacity of d.HL-60 cells was quantified using the Vybrant Phagocytosis Assay Kit (Molecular Probes). Cells were preincubated with LGG, R0011, or E. coli strain HB101 (MOI 10, 100), for 1 h at 37°C, and allowed to adhere to the bottom of 96-well plates (PerkinElmer). Supernatants were aspirated and replaced with a suspension of fluorescein-labeled E. coli (K-12 strain) in HBSS (2 h at 37°C, 5% CO2). E. coli that were not engulfed by d.HL-60 cells during this time were aspirated, and the remaining extracellular fluorescence was quenched with trypan blue (0.25 mg/ml). Intracellular fluorescence was quantified using a microplate reader equipped with filters to detect excitation/emission maxima of 480/520 nm (VICTOR X3; PerkinElmer), and the results are presented as the percentage of phagocytosis compared with untreated d.HL-60 cells.

Statistical analysis

Data are presented as mean ± SEM. Comparisons among groups of data were made using one-way ANOVA followed by Dunnett post hoc analysis. An associated probability (p < 0.05) was considered significant.

Results

LGG inhibits the formation of NETs

To investigate the efficacy of commensal, probiotic, and enterohemorrhagic bacteria to modulate the dynamics of NET formation, two neutrophil cell types were used: primary murine BMDNs and the human neutrophil–differentiated promyelocytic cell line d.HL-60. Two well-studied, gold standard activators of NETs, PMA (Fig. 1A) and the Gram-positive bacterium S. aureus (Fig. 1B, 1C), both induced the formation of NETs, as quantified by the detection of diphenylene iodinium– and DNase-sensitive extracellular DNA (SYTOX green). Although PMA induced the release of 10% of total DNA (above DNase baseline) from d.HL-60 cells and 20% of BMDNs, respectively, S. aureus promoted the release ∼20–60% of total DNA.

In the absence of stimuli, probiotic LGG lacked the ability to induce NETs. Interestingly, however, treatment of BMDNs and d.HL-60 cells with LGG (MOI 100) prior to PMA or S. aureus inhibited the formation of NETs. The requirement for LGG to be live and viable was confirmed with results showing that formalin-fixed LGG had no effect on the

Image acquisition was performed using PerkinElmer Volocity 6.2.1 software. For analysis, five random fields from each experimental treatment were captured with a 20× objective, and the total area occupied by DNA and histone H3-stained cells was quantified using ImageJ. Results are expressed as percentage cell area (per field of view) and were normalized per 100 cells.
capacity of BMDNs or d.HL-60 cells to release nuclear DNA. The absence of NETs was not due to local degradation by LGG cell wall–associated nucleases (Supplemental Fig. 1).

Immunofluorescence visualization of NETs (Fig. 2A–P), using the markers elastase and histone H3, colocalized with DNA, demonstrating that live, but not formalin-fixed, LGG inhibits the formation of these protease-rich DNA structures. In agreement with the measurement of externalized DNA using SYTOX green (above), quantification of microscopy images confirmed the inhibitory capacity of LGG on PMA-induced NETosis (Fig. 2Q).

Strain-specific ability of probiotic and enteropathogenic bacteria to activate NETs

The ability of commensal, probiotic, and enterohemorrhagic bacterial strains to induce NETosis also was assessed (Fig. 3). Although commensal E. coli strains HB101 and F18 had no effect on the capacity of BMDNs to form NETs (Fig. 3A, 3A1, 3B, 3B1), the probiotic N.1917 (Fig. 3C, 3C1) was an effective NET activator. R0011 (Fig. 3D, 3D1) did not induce NETs. Translocating C25 and enterohemorrhagic E. coli strains CL56 (but not EDL933) and adherent-invasive LF82 (Fig. 3E, 3E1, 3F, 3F1, 3G, 3G1, 3H, 3H1) also induced NET formation (quantification of microscopy images shown in Fig. 3I).

To assess their NET-inhibitory capacity, BMDNs were preincubated with the various bacterial strains and subsequently activated with PMA. Interestingly, of the bacterial strains that did not induce NETs, only LGG and R0011 had the capacity to further inhibit NET formation (Fig. 3J).

Protection against S. aureus–induced cell cytotoxicity

The induction of NETosis culminates in a time-dependent loss in cell viability, which is independent of apoptosis or necrosis (8). Although BMDNs and d.HL-60 cells challenged with PMA (60 min) induced minimal release of LDH (Fig. 4A), cells challenged with S. aureus (Fig. 4B) released significantly more LDH than did controls. This finding was most prominent in d.HL-60 cells. In line with the inhibitory effects of LGG on the formation of NETs, pretreatment of d.HL-60 cells with the probiotic protected against S. aureus–induced cell cytotoxicity, whereas formalin-fixed LGG did not rescue the cells. Of note, lactic acid production by probiotic LGG did not markedly change the pH (~7) of the reaction mixture during the incubation period with BMDNs or d.HL-60 cells (data not shown).

LGG dampens neutrophil ROS production and phagocytosis

The ability of neutrophils to generate ROS is crucial for the formation of NETs (8). Cell activation with PMA mobilized the production of ROS (such as superoxide, H₂O₂, and peroxynitrite), a process that was inhibited in the presence of live LGG but not formalin-fixed LGG. Data are mean ± SEM of at least four independent experiments. *p < 0.05 versus PMA or S. aureus alone. f.LGG, Formalin-fixed LGG.
LGG has potent antioxidative activity and protects against \( \text{H}_2\text{O}_2 \)-induced NET activation

To determine whether exogenous administration of ROS could reverse the effects of LGG, BMDNs were pretreated with either the live or formalin-fixed probiotic. However, subsequent activation with \( \text{H}_2\text{O}_2 \) failed to induce NETs in cells that had been pretreated with live LGG (Fig. 6A). Moreover, detectable ROS levels were significantly reduced (Fig. 6B), indicating that probiotic LGG has potent antioxidative properties.

Discussion

The intestinal tract serves as a reservoir for trillions of microorganisms, and the ability to discriminate and mount an appropriate host response to these microbes, whether commensal, opportunistic, or pathogenic, is essential for the maintenance of gut homeostasis. Neutrophils are abundant in the setting of intestinal inflammation, and this poses a challenging environment given the tremendous microbial burden. Despite evidence of gut pathogens, including *Shigella flexneri* (3) and *S. aureus* (8, 24), activating neutrophils to induce NETs, few studies have explored the implications of NETs on the gut microenvironment (25).

To our knowledge, we demonstrate for the first time that probiotic LGG inhibits both pharmacologic and microbial (PMA and *S. aureus*) induction of NETs. Moreover, LGG dampens ROS production and the phagocytic capacity of neutrophils, perhaps conferring a degree of hyporesponsiveness. Given the presence of NETs in inflamed intestine (3, 25), it is possible that some of the beneficial effects of LGG are attributable to its action on local neutrophils. Neutrophils produce high levels of an adenosine precursor, which, in the intestinal tract, stimulates passive water flux, providing the physiological basis of secretory diarrhea. Although NETs

FIGURE 2. Microscopy images of PMA-induced NETs in BMDNs and d.HL-60 cells. BMDNs and d.HL-60 cells were incubated in the absence or presence of LGG (MOI 100) for 1 h (37°C, 5% CO\(_2\)) on poly-l-lysine-coated glass coverslips and subsequently activated to produce NETs by exposure to PMA (100 nM, 3 h, 37°C, 5% CO\(_2\)). Cells were fixed with 4% paraformaldehyde. NETs were stained for DNA (DAPI, Invitrogen; blue), elastase (rabbit polyclonal Ab, Abcam; green) visualized with goat anti-rabbit Alexa Fluor 488 secondary Ab, and histone H3 (D1H2 XP rabbit mAb, Cell Signalling; red) visualized with tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit secondary Ab in BMDNs (A–H), and d.HL-60 cells (I–P). Preincubation with live (G, O), but not formalin-fixed (H, P), LGG inhibited PMA-induced NETosis. (E–H and M–P) Overlay of DNA and histone H3. (Q) Semiquantification of NETs in microscopy images. Images are representative of at least four independent experiments. *\( p < 0.05 \) versus BMDNs only. f.LGG, Formalin-fixed LGG.
are localized to the intestine during experimental shigellosis (3), supplementation with probiotic lactobacilli protects against *Shigella dysenteriae*–induced diarrhea in rats (26). In the clinical setting, LGG was shown to significantly reduce the duration of acute diarrheal illness in children (27).

Although NETs are essential for robust host responses to infection (7), their formation in a given microenvironment, such as the inflamed intestine, may have unintended consequences. Thus, the composition of NETs and their presence in various autoimmune and inflammatory disorders have fueled speculation regarding their roles in disease pathophysiology. Individuals with systemic lupus erythematosus and other vasculitides carry autoantibodies against neutrophil proteins (including elastase, myeloperoxidase, and lactoferrin), DNA, and histones. Indeed, such immune complexes activate NETs in vitro, and it was suggested that presentation of these Ags during NETosis likely precipitates inflammation in susceptible individuals (10). Approximately 60% of ulcerative colitis patients and 25% of Crohn’s disease patients carry autoantibodies against neutrophil proteins (28), although whether these activate NETs remains to be determined. DNase I expression, which is required for the degradation of NETs, is also reduced in inflammatory bowel disease (29). Interestingly,
long pentraxin 3, a soluble innate immune receptor of which auto-
antibodies are present in patients with systemic lupus erythematosus,
is localized to NETs and has been detected in crypt abscesses in
colic biopsies taken from patients with active ulcerative colitis (25).

The host response varies, depending on whether Gram-positive
or Gram-negative bacteria are encountered (30). Furthermore, for
a given bacterial species, strain-specific immunomodulation of
host cells has been reported (30–33). The activation of NETs by
E. coli was shown to compromise intestinal epithelial integrity (34).
Given the dynamic shift in intestinal bacterial communities during
health and disease, we also assessed the capacity of prototypical
commensal (nonpathogenic), probiotic, and enterohemorrhagic
bacteria to initiate NETosis. We show that, although commensal

FIGURE 4. LGG protects against S. aureus–induced cell cytotoxicity. LDH released from damaged cells was quantified in
BMDNs (left) and d.HL-60 cells (right) treated with PMA (100
nM, 60 min, 37°C, 5% CO₂) (A) or S. aureus (MOI 100, 60 min,
37°C, 5% CO₂) (B). Pretreatment of BMDNs and d.HL-60 cells
with LGG protected against S. aureus–induced cell damage. Data
are mean ± SEM of at least four independent experiments. *p <
0.05 versus no treatment. f.LGG, Formalin-fixed LGG.

FIGURE 5. Production of ROS is abrogated in the presence of LGG. (A) BMDNs (left) and d.HL-60 cells (right) pretreated with live, but not formalin-
fixed, LGG produced significantly less ROS in response to PMA activation, as measured by luminol chemiluminescence. (B) To quantify the effects of LGG
on superoxide levels, lucigenin was used as a chemiluminescent substrate. Live LGG (MOI 100), but not formalin-fixed LGG, inhibited superoxide
production in BMDNs and d.HL-60 cells. Superoxide production also was measured in the presence of superoxide dismutase (SOD, 30 U). *p < 0.05
versus PMA only. (C) Assessment of probiotic LGG and N.1917, or E. coli strains HB101, EDL 933, CL56, and LF82 to inhibit superoxide production in
d.HL-60 cells (MOI 100). Data are the percentage of PMA-induced superoxide production. *p < 0.05 versus PMA only. (D) Phagocytosis of fluorescein-
labeled E. coli by d.HL-60 cells was inhibited by LGG. R0011 and E. coli HB101 alone had no effect on phagocytosis. Data are mean ± SEM of at least
four independent experiments. *p < 0.05 versus d.HL-60 cells only. f.LGG, Formalin-fixed LGG.
H2O2 (10 nM). Data are mean ± SEM of at least four independent experiments. *p < 0.05 versus H2O2 only.

E. coli strains F18 and HB101 had no effect on NET formation, probiotic N.1917 was an effective NET activator. In adults, N.1917 has been used successfully for the management of Crohn’s disease and ulcerative colitis (35). However, proinflammatory effects, including potentiating of TLR4 and TLR5 activity (36), were also documented. The effects of various probiotic strains varied in their ability to inhibit NETosis, highlighting their often distinct underlying mechanisms of action (17). Proteomic analysis of LGG identified ≥50 proteins secreted into culture supernatants (37), including the soluble protein p40, which protects against cytokine-induced epithelial damage (38) and barrier dysfunction (39). Moreover, specific delivery of p40 to the mouse colon protects against colitis and epithelial cell apoptosis in an epidermal growth factor receptor–dependent manner (40). Mechanistically, we speculate that the NET-inhibitory effects of probiotic LGG may stem from an unidentified secreted factor.

R0011, like LGG, also inhibited phorbol ester–induced NETosis. However, the lack of effect on other neutrophil parameters, such as phagocytosis, suggests only a partial overlap in underlying mechanisms of action. Accordingly, although both L. rhamnosus strains secrete soluble proteins that promote intestinal epithelial homeostasis, R0011 lacks the functional pili that are present on LGG (41).

NETs can be induced by a variety of stimuli, including phorbol esters, fungi, parasites, microbial components (e.g., LPS), and ROS (42). Activation of TLR9 and platelet TLR4 induces potent NETosis (4, 6). The important role of the TLR-signaling pathway was confirmed by observations showing that neutrophils from MyD88−/− mice do not form NETs (6). Fuchs et al. (8) elegantly demonstrated time- and ROS-dependent externalization of chromatin and DNA in response to both PMA and S. aureus, two mechanistically distinct activators, which culminated in cell death independent of either apoptosis or necrosis. However, it is noteworthy that early induction of NETs by S. aureus occurs independently of ROS (24). In this study, the ability of LGG to inhibit both PKC-dependent PMA- and S. aureus–induced NETosis, while concomitantly dampening ROS production, suggests that a common downstream target of the NETosis pathway is disrupted. LGG protects against radiation-induced intestinal damage in a TLR-2/MyD88–dependent manner (43), the same pathway that is essential for mediating host responses to S. aureus (6). Interestingly, the ability of LGG to inhibit both exogenously administered ROS levels and H2O2–induced NET formation supports the antioxidative capacity of this probiotic. Indeed, some of the reported protective effects of LGG in the intestine are attributed to its modulation of oxidative parameters, including the induction of superoxide dismutase during Giardia intestinalis infection (44).

In the current study, we found that the effects of LGG on neutrophil function were also manifest by a reduced capacity of the cells to phagocytose E. coli. One of the mechanisms that pathogens use to evade neutrophils includes prevention of engulfment, through physical barriers, interference with opsonization, or inhibiting rearrangements of the F-actin cytoskeleton. It is also noteworthy that neutrophils treated with cytochalasin D, an inhibitor of actin polymerization and phagocytosis, are still able to form NETs (3).

The ability of LGG to inhibit S. aureus–induced NETs also translated to protection against cell cytotoxicity. S. aureus secretes pore-forming toxins that lyse neutrophils, including leukocidin, which was shown previously to induce the formation of NETs (24). Whether LGG secretes bacteriocins, which have antimicrobial activity against S. aureus, or directly disrupts the production of S. aureus–secreted toxins remains to be determined. Our results raise intriguing questions about the usefulness of probiotics, including LGG, in the context of infection. Indeed, Gan et al. (45) reported that Lactobacillus fermentum RC-14 and its secreted biosurfactant inhibit S. aureus infection and bacterial adherence to surgical implants.

In summary, to our knowledge, this is the first report identifying a probiotic bacterium, LGG, as an inhibitor of NETosis. We postulate that neutrophil hyporesponsiveness in a microenvironment rich in antigenic stimuli protects against the tissue-damaging properties of NETs. It is noteworthy that the inhibition of NETosis also act as a double-edged sword, given the ability of NETs to ensnare pathogens and protect against infection. However, alongside observations that LGG was able to protect against S. aureus–induced cytotoxicity, this study highlights the dynamic interaction between beneficial bacteria and neutrophils and supports the usefulness of probiotics as gut-protective factors.

Disclosures

The authors have no financial conflicts of interest.

References


Fig S1. **Nuclease activity is absent in LGG and LGG-culture supernatants.** Visualization of calf thymus DNA degradation induced by co-incubation with live bacteria (*LGG* or *S. aureus*), culture supernatants (CS) or vehicle broth without bacteria (A). Nuclease activity was present following exposure to both *S. aureus* and *S. aureus* CS, but not in response to LGG or LGG CS. Image is representative of 3 individual experiments.