

Lactobacillus reuteri DSM 17938 differentially modulates effector memory T cells and Foxp3⁺ regulatory T cells in a mouse model of necrotizing enterocolitis

Yuying Liu,¹,³ Dat Q. Tran,²,³ Nicole Y. Fatheree,¹ and J. Marc Rhoads¹,³

¹Division of Gastroenterology, Department of Pediatrics, University of Texas Health Science Center at Houston Medical School, Houston, Texas; ²Division of Allergy/Immunology/Rheumatology, Department of Pediatrics, University of Texas Health Science Center at Houston Medical School, Houston, Texas; and ³Pediatric Research Center, University of Texas Health Science Center at Houston Medical School, Houston, Texas

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Lactobacillus reuteri DSM 17938 differentially modulates effector memory T cells and Foxp3⁺ regulatory T cells in a mouse model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 307: G177–G186, 2014. First published May 22, 2014; doi:10.1152/ajpgi.00038.2014.—Necrotizing enterocolitis (NEC) is an inflammatory disease with evidence of increased production of proinflammatory cytokines in the intestinal mucosa. Lactobacillus reuteri DSM 17938 (LR17938) has been shown to have anti-inflammatory activities in an experimental model of NEC. Activated effector lymphocyte recruitment to sites of inflammation requires the sequential engagement of adhesion molecules such as CD44. The phenotype of CD44⁺CD45RBhi separates T effector/memory (Tem) cells from naive (CD44⁺CD45RBlo) cells. It is unknown whether these Tem cells participate in the inflammation associated with NEC and can be altered by LR17938. NEC was induced in 8–to 10-day-old C57BL/6J mice by gavage feeding with formula and exposure to hypoxia and cold stress for 4 days. Survival curves and histological scores were analyzed. Lymphocytes isolated from mesenteric lymph nodes and ileum were labeled for CD4, CD44, CD45RB, intracellular Foxp3, and Helios and subsequently analyzed by flow cytometry. LR17938 decreased mortality and the incidence and severity of NEC. The percentage of Tem cells in the ileum and mesenteric lymph node was increased in NEC but decreased by LR17938. Conversely, the percentage of CD4⁺Foxp3⁺ regulatory T (Treg) cells in the intestine decreased during NEC and was restored to normal by LR17938. The majority of the Treg cells preserved by LR17938 were Helios⁺ subsets, possibly of thymic origin. In conclusion, LR17938 may represent a useful treatment to prevent NEC. The mechanism of protection by LR17938 involves modulation of the balance between Tem and Treg cells. These T cell subsets might be potential biomarkers and therapeutic targets during intestinal inflammation.

NECROTIZING ENTEROCOLITIS (NEC) is the most common gastrointestinal emergency in premature infants and a leading cause of death in the neonatal intensive care unit (42). Although the pathogenesis of NEC remains incompletely defined, low birth weight, early enteral feedings, intestinal ischemic injury, and the resident bacterial flora have been identified as major risk factors in this severe inflammatory condition of the neonatal intestine (41, 42). Evidence for unregulated inflammation in NEC includes increased tissue proinflammatory mediators, including TNF-α, IL-1β, IL-6, IL-8, IL-12, and IL-18, and platelet-activating factor and increased intestinal expression of Toll-like receptor (TLR) 4 (TLR4) in patients and animals with NEC (4, 30). These data suggest that the immature neonatal intestine is prone to an exaggerated immune response to pathogenic injury.

In humans and mice, regulatory T (Treg) cells expressing the transcription factor Foxp3 are critical for immune homeostasis in the intestinal tract. In humans, patients with X-linked immune dysregulation, polyendocrinopathy, and enteropathy (IPEX syndrome), a rare condition resulting from Treg cell deficiency due to mutations in the Foxp3 gene, develop severe gastrointestinal inflammation (35), demonstrating an essential role in suppression of innate and adaptive host responses. Treg cell development can be interrupted by a local proinflammatory cytokine milieu (34, 61). A previous study attempted to investigate whether there were inadequate Treg cells in the intestine of infants with NEC. However, because of technical limitations, investigators were unable to detect a difference in the quantities of Treg cells between preterm and full-term infants when intestinal tissues were examined by immunohistochemistry (63). Recently, the same investigators performed a more detailed study using flow cytometry to quantify Treg cells in the lamina propria of resected ileum from gestational age-matched infants with and without NEC. The ratio of Treg to effector T cells was found to be significantly decreased in premature infants with NEC (63). Recently, we characterized T cell subsets in the ileum of neonatal rats with NEC by comparing these sick animals with dam-fed control rat pups. We reported that the frequency of Foxp3⁺ Treg cells was significantly diminished in NEC (27). Subsequently, we used adoptive transfer of Treg cells in this NEC model to show that increasing the number of Treg cells in the intestine improved survival and decreased the incidence and severity of NEC (11).

Postnatal gastrointestinal exposure in preterm infants differs from that in full-term infants. For example, restricted breast feeding and treatment with antibiotics may lead to abnormal bacterial colonization and altered immunological development in the gut, thereby increasing the susceptibility to NEC (7, 38, 48). T cells are present in the human fetal ileum in early gestation and accumulate during chorioamnionitis (53). Single-nucleotide polymorphism studies of genetic risk factors for NEC suggest that an enhanced

Address for reprint requests and other correspondence: J. M. Rhoads, Dept. of Pediatric-GI, The Univ. of Texas Health Science Center at Houston Medical School, 6431 Fannin St., MSB.3.137, Houston, TX 77030 (e-mail: j.marc.rhoads@uth.tmc.edu).

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Th1-mediated immune response is associated with more severe disease (60). In the pathogenesis of NEC, the balance of immune regulation in the intestinal mucosa might be disrupted not only by a deficiency of Treg cells, but also by the presence of an expanded effector T cell population that reacts to commensals or pathogens.

Activated effector lymphocyte recruitment to sites of inflammation requires the sequential engagement of adhesion molecules such as CD44. Adhesion receptor CD44 is associated with diverse biological processes involving the migration of cells, including inflammation, angiogenesis, bone metabolism, and wound healing (2). CD44 is upregulated concomitant with the activation of naive T lymphocytes during invasive microbial infection (2). Recent studies showed that CD44 deficiency attenuates murine ileitis (8). The adhesion molecule CD44, as a marker for activated effector CD4+ T cells in inflamed tissues, potentiates T cell activation and maintenance of T cell memory function (3). The CD45 isoform CD45RB (restricted to exon 5 (B)) (33) has been shown to be highly expressed on memory function (3). The CD45 isoform CD45RB is restricted to naive (CD44- CD45RB+) cells in the non-Treg cell population (57). It is not clear whether Tem cells residing in the intestinal mucosa play a role in NEC.

Probiotic supplementation has been associated with a significantly decreased risk of NEC in preterm very-low-birth-weight infants (62). Lactobacillus reuteri DSM 17938 (LR17938) was derived from L. reuteri ATCC 55730, a component of a Peruvian mother’s breast milk. Two plasmids harboring antibiotic resistance genes were removed from strain 55730 to obtain strain 17938 (46), which inhibits pathogen growth and modulates the immune system. We have shown that feeding LR17938 to newborn rats produced a strong anti-inflammatory effect (28, 29), reducing the incidence and severity of NEC (29) while increasing the frequency of Treg cells in the intestinal mucosa (27).

The aim of this study was to identify changes in Tem and Treg cells in the intestine of mice with NEC, thereby clarifying the immunomodulatory mechanism of LR17938 in a mouse model of NEC. We determined if the frequency of Tem or Foxp3+ Treg cells changes in the intestine and mesenteric lymph nodes (MLNs) of mice with NEC, and we determined if changes could be reversed by LR17938 supplementation.

MATERIALS AND METHODS

Probiotic LR17938 Preparation

Human breast milk-derived LR17938 was provided by Biogaia (Stockholm, Sweden). Lactobacillus acidophilus DDS (La DDS; kindly provided by Dr. David R. Mack, Children’s Hospital of Eastern Ontario, Ottawa, ON, Canada) was used as the control bacterium for immune cell analysis. Lactobacillus organisms were anaerobically cultured in deMan-Rogosa-Sharpe (MRS) medium (Difco, Detroit, MI) at 37°C for 24 h, plated in MRS agar at specific serial dilutions, and grown anaerobically at 37°C for 48–72 h. For quantitative analysis of bacteria in culture medium, a photometer (Eppendorf, Hamburg, Germany) was used to compare absorbance (at 600 nm) of culture at known concentrations using a standard curve of bacterial colony-forming units (CFU) per milliliter grown on MRS agar. Bacteria in the culture medium were harvested by centrifugation at 1,500 g for 15 min and resuspended in formula before feeding.

Experimental NEC Model and Experimental Design

Animal studies were approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston (HSC-AWC-11-063). Experimental NEC model. We developed a protocol for NEC induction in mouse pups that included formula feeding + exposure to stress (FS); this model was not associated with excessive early mortality. We modified the techniques of Jilling et al. (23), Nadler et al. (40), and Sodhi et al. (51). Neonatal (8–to 10-day-old) C57BL/6J mice from breeding pairs of adult male and female animals (Jackson Laboratory) were separated from their dams, housed in an incubator, and starved for 12 h before the initiation of orogastric feeding with 0.2 ml of formula via a sterile Solomon 22-gauge 35-mm feeding needle (Instech Laboratories) four times daily for 4 days. To induce NEC, mouse pups were exposed to 5% O2-95% N2 for 10 min in a hypoxia chamber (Billups-Rothenberg, Del Mar, CA) and then to cold stress at 4°C for 5 min twice daily for 4 days. The formula consisted of 15 g of Similac 60/40 (Ross Pediatrics, Columbus, OH) in 75 ml of Esbilac canine milk replacement (PetAg, Hampshire, IL), which contains 1.86 kcal/ml. The calculated calorie intake met the maintenance energy requirement of newborn mice (~200 kcal·kg⁻¹·day⁻¹). Animals were monitored every 3 h during the 4-day study period. No analgesia was necessary for mice or rats in this study or in previous publications by other groups (1, 17, 23, 45). Live animals were counted on each day, and pups were euthanized on day 3 after NEC induction (FS) for tissue collection. In some cases, pups in the FS group were euthanized on day 3 or 4, for example, if they were in pain, demonstrated labored respirations, exhibited abdominal distension, or had gastrointestinal bleeding. After euthanization, we collected tissues for histological analysis.

Experimental groups. Mouse pups were randomly divided into four groups: 1) dam-fed mouse pups [normal control (dam), n = 23] were left with their mothers to breast-feed; 2) dam + 17938 (10⁶ CFU·g body wt⁻¹·day⁻¹) mouse pups (n = 16) were left with their mothers to breast-feed and were given LR17938 by gavage for 7 days; 3) formula + stress (FS) mouse pups (n = 36) were separated from their mothers, housed in an incubator, fed formula, and exposed to stress (hypoxia and hyperthermia) for 4 days to induce NEC; and 4) formula + stress + 17938 (FS + 17938) mouse pups (n = 20) were treated as described for the FS group, except the formula contained LR17938 (10³ CFU·g body wt⁻¹·day⁻¹). For characterization of Tem and Treg cells, we also added one group of FS + La DDS mouse pups (n = 4), which were treated as described for the FS group, except the formula contained La DDS (10³ CFU·g body wt⁻¹·day⁻¹).

Tissue Harvest and NEC Evaluation

After incision of the abdomen, the gastrointestinal tract was carefully removed. The small intestine was evaluated visually for typical gross signs of NEC, such as intestinal distension, wall hemorrhage, or necrosis. The terminal 5 cm of small intestine (ileum) were excised. The sections of ileal tissues were prepared longitudinally and stained with hematoxylin and eosin for histological evaluation. The remaining 4 cm of small intestine were used for isolation of lymphocytes. In some cases, pups in the FS group were euthanized on day 3 after NEC induction (FS) for tissue collection. In some cases, pups in the FS group were euthanized on day 3 or 4, for example, if they were in pain, demonstrated labored respirations, exhibited abdominal distension, or had gastrointestinal bleeding. After euthanization, we collected tissues for histological analysis.

Tissue Preparation for Cytokine IL-1β Assay

Ileal tissues were weighed and then homogenized in 0.4 ml of lysis buffer containing protease inhibitors with 20 mmol/l Tris-HCl (pH
L. reuteri DSM 17938 AFFECTS EFFECTOR/MEMORY T AND Treg CELLS

L. reuteri DSM 17938 Reduced the Incidence and Severity of NEC

The severity of NEC was evaluated by histological NEC scoring of ileal tissues on a scale of 1–3 (1, 5). Animals with histological scores ≥2 were defined as having NEC. Intestinal tissues for evaluation of histological changes were obtained from living animals on day 5. However, in some groups with severe NEC, tissues were collected on day 3 or 4 for the purpose of comparison. Morphological analysis of ileal segments from mice subjected to the NEC protocol revealed various degrees of inflammatory change, ranging from sloughing of epithelial cells to the midvillous level to necrosis of the entire villus (Fig. 2A), and villous core separation was also seen (Fig. 2A, red arrow). For comparison, Fig. 2A shows the histological features of ileal mucosa from dam-fed and FS + 17938 animals. In contrast, dam-fed and dam-fed + LR17938 mice had normal intestinal architecture and rarely showed inflammatory changes. The incidence of histological NEC was significantly higher in the FS group (11 of 17, 65%) than in dam-fed (0 of 23) or dam-fed + 17938 (0 of 16) mice (P < 0.01).

Feeding LR17938 to mice subjected to the NEC protocol significantly reduced the incidence of histological NEC to 27% (4 of 15) compared with 65% in the FS group (11 of 17, P < 0.05; Fig. 2B). In addition, the severity of NEC also decreased with administration of LR17938, demonstrating that probiotic administration shifted intestinal damage from severe to mild, as indicated by a change in the NEC score from 2 or 3 to 1. Specifically, 29% (5 of 17) had a NEC score of 3 in the FS group compared with 13% (2 of 15) in the FS + 17938 group (P < 0.05); 35% (6 of 17) had a NEC score of 2 compared with...
Fig. 2. LR17938 reduced incidence and severity of NEC. A: representative intestinal histological changes in dam-fed, dam-fed + 17938, FS (NEC protocol), and FS + 17938 mice. Ileal tissues were stained with hematoxylin and eosin. Magnification ×200. Red arrows, villous core separations in animals with NEC; black arrows, normal villi in ileal tissue from dam-fed or FS + 17938 mice. B: number of animals with normal histology (NEC scores ≤2) and with NEC (NEC scores ≥2). n, Number of live animals from which tissue samples could be collected. Terminal ileum of each mouse was harvested at day 5 (or day 3 or 4 for some samples in the FS group). %. Incidence of NEC. *P < 0.05 vs. FS. C: FS and FS + 17938 groups with NEC scores of 2 and 3. *P < 0.05 vs. FS. LR17938 reduced NEC histological injury.
13% (2 of 15) in the FS + 17938 group (P < 0.05; Fig. 2C). Conversely, a score of 0 or 1 was seen in 73% (11 of 15) of animals in the FS + 17938 group compared with 35% (6 of 17) of pups in the FS group.

*L. reuteri* DSM 17938 Decreased Inflammatory Cytokine IL-1β Levels in the Intestine of Mice With NEC

Cytokines are key mediators in inflammation, and several cytokines, including IL-1β, are dysregulated in this disease (30, 50). Analysis of intestinal protein levels of IL-1β indicated that this proinflammatory cytokine significantly increased in the intestines of mouse pups given the NEC-inducing FS treatment compared with normal dam-fed mice (P < 0.01), while feeding LR17938 to mice with FS significantly decreased IL-1β levels compared with FS without probiotic treatment (P < 0.05; Fig. 3).

Frequency of Treg Cells in the Intestinal Mucosa Was Decreased in NEC and Could Be Reversed by Feeding *L. reuteri* DSM 17938

Treg cells maintain intestinal homeostasis by controlling inflammation and inducing tolerance. In a previous study we proposed that there are insufficient numbers of Treg cells to control inflammation in NEC on the basis of a decreased frequency of Treg cells in the intestines of rat pups with NEC (27). In the present study we further validated this observation in the mouse model of NEC by analyzing the Treg cell counts by gating CD4+ T cells to calculate the frequency of Foxp3+ Treg cells within the CD4+ T cell population (Fig. 4A).

The frequency of CD4+Foxp3+ Treg cells significantly decreased in the ileum (Fig. 4B) and MLNs of FS-exposed mice (Fig. 4C) compared with normal dam-fed mice (P < 0.01 in the ileum; P < 0.001 in the MLN). In response to LR17938, the frequency of CD4+Foxp3+ Treg cells returned to normal in the ileum (P < 0.001), but not in the MLNs, compared with age-matched dam-fed control animals without probiotic (Fig. 4D). However, feeding *Lactobacillus acidophilus* DDS, unlike *L. reuteri* (LR17938), could not...

![Figure 3](image-url)  
Fig. 3. Increased IL-1β levels in intestine of mice subjected to the NEC protocol and the modifying effect of LR17938 treatment. Cytokine levels in tissue lysates were examined using Meso Scale Discovery cytokine assay; n = 8–12 mice per group. Comparisons showed significant differences: P < 0.01 (FS vs. dam) and P < 0.05 (FS + 17938 vs. FS).

![Figure 4](image-url)  
Fig. 4. LR17938 reversed frequency of CD4+Foxp3+ regulatory T (Treg) cells in terminal ileum and mesenteric lymph nodes (MLNs) of mice with NEC. A: representative flow cytometric plots from terminal ileum of dam-fed mice with initial gating on lymphocyte population on the left forward scatter (FSC)-side scatter (SSC) plot (left), followed by gating on CD4+ T cells [middle; x-axis: CD4-peridinin-chlorophyll proteins (CD4-PerCP/Cy5.5); y-axis: CD–phycoerythrin (CD8-PE)], and Foxp3+ Treg cells within the group of CD8−CD4+ T cells (right; x-axis: CD4-PerCP/Cy5.5; y-axis: Foxp3-Alexa Fluor 647). B and C: frequency (%) of CD4+Foxp3+ Treg cells in terminal ileum and MLNs. n, Number of live animals from which sufficient cell numbers were obtained from tissues for analysis. FS vs. dam: P < 0.01 (B) or P < 0.001 (C); FS vs. FS + 17938: P < 0.01 (B and C); FS vs. FS + *Lactobacillus acidophilus* DDS (La DDS; B and C): P > 0.05. D: percentage of Treg cells in terminal ileum of dam-fed + LR17938 and dam-fed mice (P < 0.001).
increase the frequency of CD4⁺Foxp3⁺ Treg cells in the ileum or MLNs of mice subjected to NEC-inducing conditions (Fig. 4, B and C).

Helios⁺Foxp3⁺ Cells Were the Dominant Treg Cell Subset in the Ileum After LR17938 Administration to Mice With NEC

After demonstrating that CD4⁺Foxp3⁺ Treg cells in the ileum of mice exposed to the NEC-inducing procedure were augmented by feeding LR17938, we took advantage of a new marker called Helios, which is reported to detect Treg cells of thymic origin in mice (58). We found that 70% (68 ± 2.8%, n = 23) of CD4⁺Foxp3⁺ Treg cells in the ileum of dam-fed mice expressed Helios (Fig. 5). Helios was undetectable in the ileum of FS-exposed mice because of the significantly low levels of CD4⁺Foxp3⁺ Treg cells. However, ~75% (76 ± 4.4%, n = 11) of Treg cells expressed Helios in the ileum of FS-exposed mice that were fed LR17938, indicating that the increased CD4⁺Foxp3⁺ Treg cells in mice could be of thymic origin. In the MLNs, too, 75–80% of CD4⁺Foxp3⁺ Treg cells showed Helios positivity. There were no significant differences between the groups.

Tem Cells in the Intestinal Mucosa Were Elevated in NEC and Decreased by Feeding LR17938

Tem cells express a different pattern of cell surface markers, and functionally they respond in several different ways compared with naïve T cells. In mice, it has been noted that activation of lymphocytes and the transition to memory/effector phenotypes are associated with increased surface levels of CD44 (25). CD44, as an adhesion molecule, is required for recruitment of activated effector lymphocytes to sites of inflammation (2). In addition, naïve T cells express a high level of the CD45 isoform restricted to exon 5 (B), named CD45RB in mice. Loss of CD45RB expression has been demonstrated when naïve T cells change to Tem cells. Therefore, the phenotype of CD44⁺CD45RB⁻ separates Tem from naïve (CD44⁺CD45RB⁺) cells (59). The Tem cells are required for immunological protection, while Foxp3⁺ Treg cells are needed to restrain the immune system from excessive inflammation and/or autoimmunity (31). Thus both cell types are extremely important for the maintenance of immunological homeostasis in the host.

Because our studies indicated that the frequency of Foxp3⁺ Treg cells was decreased in NEC, we queried whether Tem cells could play a role in NEC. We stained immune cells isolated from the ileum and MLNs with antibodies to CD4, CD44, CD45RB, and Foxp3 and analyzed the cells by flow cytometry. After gating CD4⁺ T cells, populations of Foxp3⁺ Treg cells and Foxp3⁻ non-Treg cells were defined, and the percentages of Tem cells (CD44⁺CD45RB⁻) and transitional effector T (CD44⁺CD45RB⁺) cells among the non-Treg cell population were analyzed (Fig. 6A). A significant increase in the percentage of Tem cells in the intestine was observed in mice exposed to NEC conditions (12.1 ± 0.9%, n = 14) compared with dam-fed controls (3.3 ± 1.9%, n = 23, P < 0.001). Feeding LR17938 to FS-exposed mice significantly decreased the Tem subset (6.1 ± 0.6%, n = 11) compared with samples from mice exposed to FS without probiotic (P < 0.001). We were surprised to discover that administration of LR17938 to healthy dam-fed mice also increased the Tem cell subset (6.8 ± 0.7%, n = 16) compared with dam-fed controls (P < 0.01). Feeding the “control” Lactobacillus La DDS could not change the increased percentage of Tem cells in the intestine induced by the NEC procedure (Fig. 6B). A similar pattern was observed in the percentage of transitional effector (CD44⁺CD45RB⁻) cells in the intestine (Fig. 6C), with increased expression in FS-exposed animals and reduced expression in the FS + LR17938 group. There were no changes in these two T cell subsets in MLNs among the different groups, indicating that the changes in Tem cells were limited to the intestine.

DISCUSSION

NEC is a devastating disease of neonates associated with high morbidity and mortality (14). Over the past 15 years, a number of studies have investigated the effects of probiotics in preventing NEC (62). LR17938 inhibits pathogen growth and modulates the immune system. In this study the protective effects of the probiotic LR17938 on the survival, incidence, and severity of NEC in a mouse model further validated our previous observations in a rat NEC model (27, 29). In addition, previous studies indicated that the frequency of gut Foxp3⁺ Treg cells was decreased in NEC but reverted to normal following LR17938 treatment (27).

Central features of NEC pathophysiology include immaturity of the intestinal barrier and aberrant mucosal immunity, leading to bacterial invasion and markedly exaggerated inflammation in response to bacterial antigens. Supporting evidence includes higher serum and intestinal levels of several cytokines and chemokines during NEC (30, 50). Furthermore, increased TLR signaling activity in the intestine of rats with NEC has been demonstrated (16, 29–31, 52). We believe that LR17938 could prevent NEC via modulation of TLR4 and NF-κB signaling in the intestine (29).

Foxp3⁺ Treg cells are essential for intestinal immune homeostasis through suppression of innate and adaptive host responses. We proposed that disrupted immune regulation may be involved in perturbing the balance between Treg and Tem cells. CD4⁺ T cells express high levels of CD44, which is
involved in Th1-driven inflammation (39), including recirculation of T cells to inflammatory sites and chemotaxis (8). CD44 extracted from Th1 cells was found to bind soluble E-selectin in vitro and cooperated with P-selectin glycoprotein ligand-1 in mediating a rolling interaction between T lymphocytes and the vascular endothelium. A combined absence of CD44 and P-selectin glycoprotein ligand-1 impairs inflammatory T cell recruitment (39). CD44\(^{+}\)CD45RB\(^{-}\) T cells were found to have effector function and phenotype, to show enhanced reactivity to CD44 ligand hyaluronan, to produce inflammatory cytokines such as TNF-\(\alpha\) and IL-2, and to be essential for the induction of ileitis in RAG\(^{-/-}\) mice (8). Conversely, CD44 deficiency attenuated chronic murine ileitis (8). In addition to expressing CD44, the majority of effector/memory cells express low levels of CD45RB and L-selectin (59). The adoptive transfer of lamina propria CD44\(^{hi}\)CD62L\(^{lo}\)CD45RB\(^{-}\)Tem cells into severe combined immunodeficiency mice induced a severe colitis (15). However, it was not clear whether this Tem cell subset in the intestinal mucosa is involved in the pathogenesis of NEC.

On the opposite side of these changes, our previous study with adoptive Treg cells in a rat model of NEC demonstrated the ability of Treg cells to attenuate the development of NEC by inhibiting the activation of dendritic cells and the maturation of naive CD4\(^{+}\)CD62L\(^{+}\) into effector CD4\(^{+}\)CD62L\(^{-}\) cells (11). Our current studies in a mouse model of NEC further confirm the loss of balance between these two T cell subsets in the intestinal mucosa, demonstrating increased intestinal Tem and transitional effector T cells in the face of decreased Foxp3\(^{+}\) Treg cells during NEC (compared with dam-fed controls). Treg cell development and function can be interrupted by a local proinflammatory cytokine milieu (9, 24). During the development of human or experimental NEC, formula feeding or stress may increase levels of endogenous TLR ligands (e.g., heat shock protein) in the intestinal inflamed tissue (20, 22), which, in combination with exogenous TLR ligands (e.g., bacterial lipopolysaccharide), would serve to activate TLRs and TLR-signaling pathways to produce proinflammatory cytokines by intestinal epithelial and immune cells (26, 30).

Finally, we found that LR17938 ingestion produced a “rebound” of intestinal Foxp3\(^{+}\) Treg cells and decreased Tem cells not only in the intestine but also in the MLNs of mice with NEC. This differential modulation of LR17938 on Tem and Treg cells was not observed when animals were treated with the control Lactobacillus strain La DDS, most likely because La DDS has been shown to lack the properties of adherence to epithelial cells, induction of mucin expression by intestinal epithelial cells, inhibition of enteropathogenic Escherichia coli epithelial cell adherence (32), and inhibition of LPS-induced NF-\(\kappa\)B activation (29). In contrast, L. reuteri ATCC 55730 (a parent strain of LR17938) has been shown to adhere to Caco-2 human intestinal epithelial cells (37). Recent studies indicated...
that this L. reuteri strain possesses a gene encoding a protein (MapA) or a collagen-binding protein (CnBP) (49). These findings further support adhesion mechanisms in the probiotic-host interaction. In addition, L. reuteri strains inhibit enteropathogenic E. coli by direct antimicrobial activity attributable to secreted reuterin (54) or to the repression of genes expressed by pathogenic E. coli (21).

We further studied the subset of these Foxp3+ Treg cells based on their expression of Helios. While controversial, it has been suggested that Helios can be a marker of thymus-derived Treg (tTreg) cells, and those lacking Helios would represent peripheral induced Treg (pTreg) cells (10). The tTreg and pTreg cells play nonredundant roles in immune regulation, but their functions are not easy to distinguish, in part because we lack markers to unambiguously discriminate between the two cell types. It has been proposed that only tTreg cells express Helios and neutropelin-1, which are expressed by most Foxp3+ Treg cells in blood and in the thymus (43). We therefore analyzed the expression of Helios within the CD4+ Foxp3+ population in the ileum. We found that 75% of CD4+ Foxp3+ Treg cells express Helios in the NEC-exposed group treated with probiotic LR17938, approximately the same level as seen in dam-fed controls. This finding indicates that the increased Treg cells in mice fed the probiotic were likely of thymic origin. In the MLNs, ~70–80% of Treg cells were Helios+ tTreg cells in all studied groups. Our observations may be seen as contrasting with a previous report, in which the intestinal lamina propria Treg cell pool appeared to be enriched in pTreg cells (43). We speculate that the immunological difference might be due to observations in newborn vs. mature mice. Further study of molecules that mediate T cell homing to the gut, including the chemokines (e.g., CCL25), chemokine receptors (e.g., CCR9), integrins, and ligands (e.g., β7-integrin and mucosal addressin cell adhesion molecule-1) (44, 55), is needed in newborn subjects.

Finally, our current studies also showed that breast milk leads to increased intestinal Treg cells, which implies that some factor(s) in breast milk may enhance the immunomodulatory effects of LR17938. It has been known that breast milk contains bioactive milk proteins, which act against the inflammatory process, suggested by in vitro and in vivo studies (6). Breast milk contains casein and whey proteins (including α-lactalbumin, β-lactoglobulin, and osteopontin) that are believed to favor the growth of certain bacterial species (36), whereas whey proteins and IgA directly or indirectly modulate the immune system. Human milk contains up to 10^9 CFU/ml live bacteria, with a predominance of Bifidobacteria (18). The protective effect of maternal milk is associated with increased production of mucosal IL-10 in the site of injury, which indicates an immunomodulatory activity (12). Maternal milk is the major source of epidermal growth factor for neonates, which plays an important protective role against NEC development (13). Transforming growth factor (TGF)-β2, an immunomodulatory growth factor, is the most dominant form of the TGF-β superfamily in breast milk. The intestinal immune system in preterm neonates is immature, producing remarkably low amounts of endogenous TGF-β and TGF-β receptor (47). The restoration of these low levels of TGF-β through feeding breast milk is pivotal for provision of adequate protection against inflammation. Insulin-like growth factors (IGFs), such as IGF-I and IGF-II, important growth factors in breast milk (56), may initiate intracellular signaling, suppressing apoptosis and stimulating cell proliferation. The interaction of these factors with intraluminal LR17938 during immunomodulation should be further explored.

In summary, our data indicate that 1) NEC is an inflammatory condition with dysregulated immune activation based on increased Tem and decreased Treg cell activities; 2) L. reuteri DSM 17938 during NEC appears to promote Treg cell development and/or migration and to decrease Tem cells to dampen immune activation; and 3) the majority of the Treg cells associated with LR17938 treatment are of the Helios+ subset, which suggests a systemic effect on gut inflammation in NEC wherein thymus-derived circulating Treg cells might be attracted to the injured intestine. L. reuteri may directly affect the expression and activation of the chemotactant molecules in the intestinal mucosa and/or may indirectly affect these molecules by inducing expression of higher levels of retinoic acid-producing enzymes on gut dendritic cells, which trigger the expression of gut-homing molecules (19). We acknowledge that a prophylactic, not a therapeutic, effect was demonstrated in these studies once the inflammation developed. The question of a therapeutic effect requires further studies. Our results support the concept that probiotic L. reuteri DSM 17938 may represent a useful treatment to prevent NEC. In addition, these T cell subsets might be potential biomarkers and therapeutic targets during intestinal inflammation.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.L., D.Q.T and J.M.R. are responsible for conception and design of the research; Y.L. and N.Y.F. performed the experiments; Y.L. and D.Q.T. analyzed the data; Y.L., D.Q.T. and J.M.R. interpreted the results of the experiments; Y.L. prepared the figures; Y.L. drafted the manuscript; Y.L., D.Q.T. and J.M.R. edited and revised the manuscript; J.M.R. approved the final version of the manuscript.

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