

# Cellular strain amplifies LPS-induced stress signaling in immature enterocytes: potential implications for preterm infant NCPAP

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**BACKGROUND:** Recent clinical observations of increased necrotizing enterocolitis (NEC) incidence in some nasal continuous positive airway pressure (NCPAP) patients raise concerns about whether the related abdominal distension is benign or contributes to NEC. We tested the hypothesis that mechanical strain causes an exaggerated enterocyte inflammatory response and decreased enterocyte growth and proliferation in the absence and presence of lipopolysaccharide (LPS).

**METHODS:** First we used a confluent enterocyte (IEC-6) monolayer to investigate effects of strain on inflammatory cytokine production and Toll-like receptor 4 (*TLR-4*) gene expression. Then we used a low seeding density to measure cell growth and proliferation. Ten percent mechanical strain was applied.

**RESULTS:** Significant increases in interleukin (IL)-8 and in IL-6 were observed after 8 and 24 h of cellular strain, respectively, and maintained throughout the study. *TLR-4* expression was increased at 48 h. Mechanical strain led to slower proliferation and division whereas LPS alone had minimal effects. The responses of LPS and strain were supra-additive, suggesting synergistic cellular effects.

**CONCLUSION:** We speculate intestinal distension associated with the use of NCPAP, especially in the presence of abnormal gut colonization, may result in increased inflammatory cytokine production and be a contributing factor to neonatal intestinal morbidities.

Infants born prematurely often require some form of respiratory support in the first weeks of life, and one such approach is the use of nasal continuous positive airway pressure (NCPAP). This system improves gas exchange through dilation of the respiratory tree (via increased pressure through the nasal pharynx) and prevention of expiratory lung collapse, thereby decreasing respiratory effort and improving oxygenation. Its use in premature infants is important because of the difficulty these newborns have with maintaining upper airway patency and functional residual capacity (1). As compared with the use of mechanical ventilators, NCPAP facilitates early extubation and has decreased incidence of acute lung injury and related complications (2–5). Observational studies have also shown

that the use of NCPAP in place of mechanical ventilation has resulted in a reduction of oxygen dependence for preterm infants at 28 d (2,4). Collectively, these findings have promoted a transition to the use of NCPAP within most neonatal intensive care settings wherever feasible.

Although NCPAP has definite benefits with respect to pulmonary and oxygenation status in preterm infants, its use is also associated with abdominal distension, commonly referred to as “CPAP belly syndrome.” This abdominal distension has been observed to occur ~4–7 d following initiation of NCPAP and involves dilation of both the small and large bowel (6). It has been suggested that CPAP belly syndrome is related to the functional immaturity and poor motility of the intestine and trapping of air driven by positive pressure at the nasal cavity. The condition appears to be most severe in the smallest and most premature infants, as Jaile *et al.* (6) noted that abdominal distension occurred within 83% of infants weighing <1,000 g, but only in 14% of infants weighing >1,000 g. In this patient population, NCPAP can cause gastrointestinal air-trapping and distended abdominal circumference by as much as 30% (P.J. Giannone, B.L. Schanbacher, J.A. Bauer *et al.*, unpublished data). Given the rapidly expanded use of NCPAP in neonatal nurseries, and the increasing prevalence of preterm births worldwide, a closer study of this gastrointestinal challenge in the youngest of patients appears warranted, and thus far the findings are controversial. For example, Jaile *et al.* (6), found no increase in gastrointestinal issues for those infants who developed abdominal distension on NCPAP, suggesting that CPAP belly syndrome is a benign entity. In contrast, Aly *et al.* (3) found an association of NCPAP use and increased incidence of necrotizing enterocolitis (NEC), the most common gastrointestinal disease that afflicts newborn infants. Approximately 5–10% of all infants with a birth weight ≤1,500 g are diagnosed with NEC, and this is associated with a mortality rate of 15–30% (7,8). The mechanisms of NEC are not clearly defined, but specific risk factors include prematurity, ischemia, aggressive enteral feedings, and infection; inflammation-driven tissue injury and necrosis are predominant mechanisms (9–11). The vulnerability of premature infants to the development of

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NEC has been attributed to an immature gastrointestinal tract vulnerable to potential overgrowth of pathogens, especially as a result of poor motility (10,12).

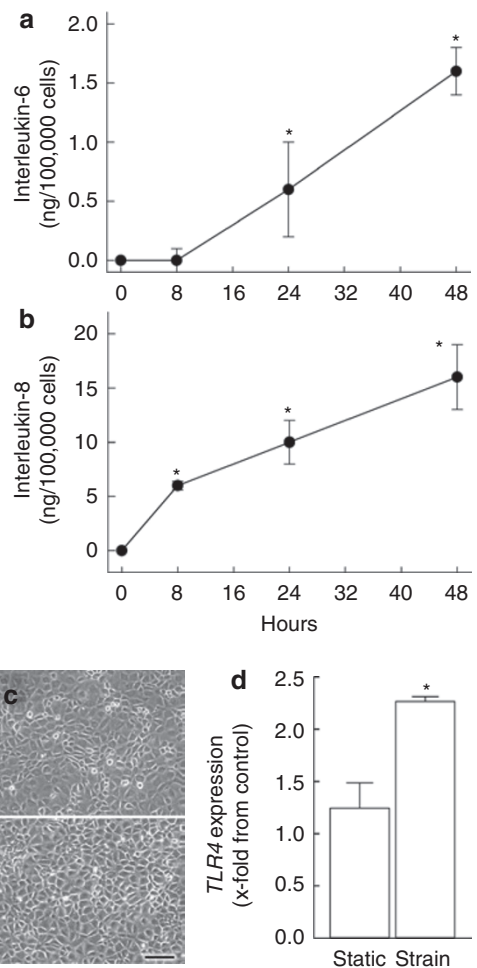
Recent clinical observations of increased NEC incidence in some NCPAP patients raise concerns about whether the related abdominal distension is benign in all infants, or if it contributes to NEC pathogenesis. This may be of special concern in the extremely premature, wherein abdominal wall tone and intestinal maturation are both minimal and organ distension may be most extensive. To our knowledge, no observational or randomized trial study has been conducted to specifically address potential differences in the occurrence or progression of NEC with the use of NCPAP. Furthermore, the potential interactions of intra-luminal intestinal pressure and other stimuli that are suspected contributors to intestinal injury and risk factors for NEC have not been investigated. For these reasons we studied the effects of mechanical strain on isolated immature intestinal epithelial cells *in vitro*. Our objective herein was to test the hypothesis that mechanical strain causes an exaggerated enterocyte inflammatory response, in the absence and presence of inflammatory stimulation via lipopolysaccharide (LPS).

## RESULTS

Initial studies were conducted to determine the optimal starting conditions for the neonatal rat enterocytes to adequately adhere before applying strain. We found that seeding with at least 250,000 cells/cm<sup>2</sup> of membrane surface, and allowing a 48-h post-seeding time before applying strain reliably provided fully confluent cell monolayers. Preliminary investigations also showed that the monolayer was maintained throughout the cyclic strain conditions we employed (20% distension, 3 s/cycle, 10 cycles/min) with at least 90% confluence throughout the study period.

Time-dependent effects of mechanical strain on production of inflammatory cytokines by already confluent neonatal rat intestinal epithelial cells are shown in **Figure 1**. Statistically significant increases in interleukin (IL)-6 (**Figure 1a**) and in IL-8 (**Figure 1b**) were observed after 24 and 8 h of cellular strain, respectively, and this was maintained throughout the remainder of the 48 h studied. The increases in IL-8 were found to be more extensive than those in IL-6, with nearly 10-fold higher levels of IL-8 from this cell type in response to mechanical strain. Under the conditions employed the total number of adherent cells was not different from that of the control treatment (e.g., cells cultured on identical membranes but with no strain forces applied, see (**Figure 1c**)). Significantly increased gene expression of Toll-like receptor 4 (*TLR-4*) was also observed in strained enterocytes after 48 h as compared with static control conditions (**Figure 1d**).

Concentration- and time-dependent changes of IL-6 and IL-8 during LPS exposure to rat neonatal enterocytes under static conditions (e.g., in the absence of strain) are shown in **Figure 2**. Statistically significant increases in both cytokines were observed by 24 h of treatment and were maintained until 48 h. In relative comparison, the LPS-concentration response

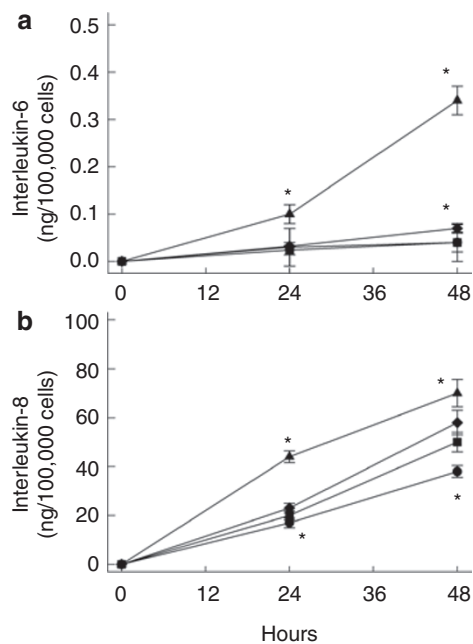


**Figure 1.** Effects of cellular strain alone on rat neonatal enterocytes. (a) Time-dependent increases in interleukin (IL)-6. (b) Time-dependent increases in IL-8. (c) Representative images of static (upper panel) vs. strained enterocyte monolayer following 48 h. Scale bar = 100  $\mu$ m. (d) Increased Toll-like receptor 4 gene expression following 48 h of strain vs. static conditions. All panels,  $n = 6-12$ , mean  $\pm$  SE; \* $P < 0.05$  as compared with time 0 static control.

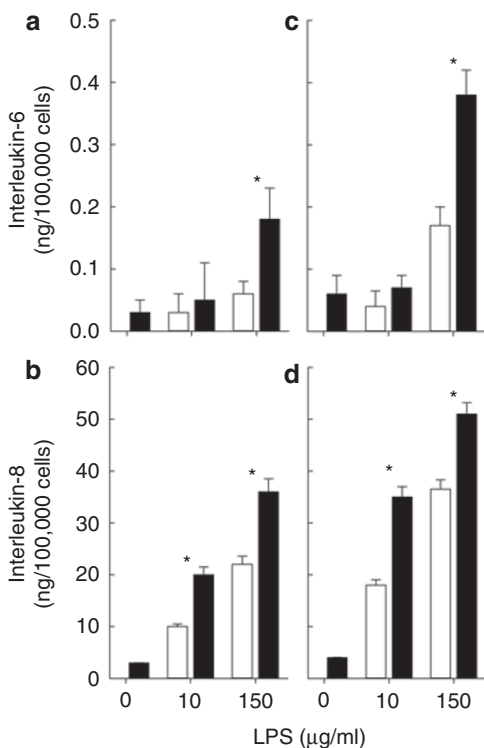
was more robust with respect to IL-8 expression than IL-6 expression under these conditions.

Synergistic effects of cellular strain and LPS exposures with respect to cytokine expression by confluent rat neonatal enterocytes are shown in **Figure 3**. Confluent enterocyte monolayers were exposed to cyclic strain, LPS, or both for 24 and 48 h. LPS caused time-dependent increases in IL-6 and IL-8 production at 10 and 150  $\mu$ g/ml; these LPS responses were substantially increased when combined with the presence of cellular strain. In most cases (with the exception of 10  $\mu$ g/ml LPS and IL-6 response) the response to the combination of LPS and strain was supra-additive as compared with the two stimuli used alone; suggesting that this combined challenge caused synergistic cellular effects.

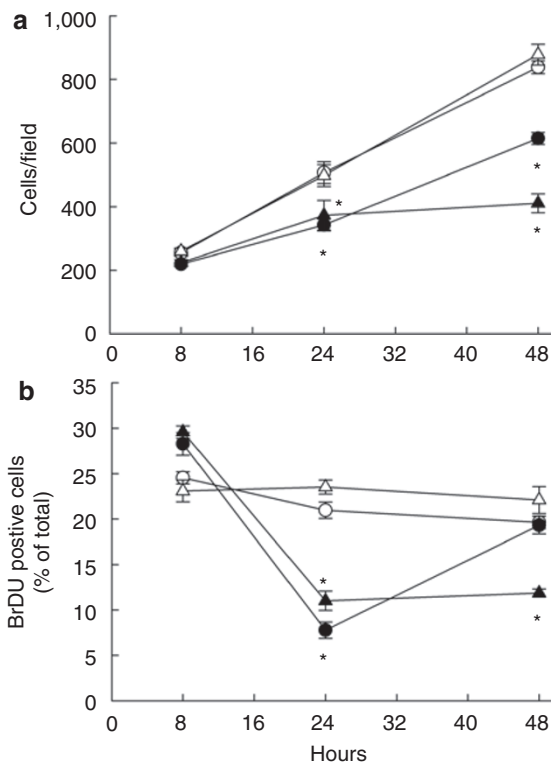
The effects of cellular strain, LPS, or their combination on enterocyte growth and proliferation before confluence are shown in **Figure 4**. At fixed time points, numbers of cells per field of view were recorded (**Figure 4a**), and actively dividing



**Figure 2.** Effects of lipopolysaccharide (LPS) alone on rat neonatal enterocytes. Time-dependent increases in (a) interleukin (IL)-6 or (b) IL-8 during various LPS exposures ( $n = 6-12$ , mean  $\pm$  SE) (filled circle = 10  $\mu$ g/ml, filled square = 50  $\mu$ g/ml, filled triangle = 150  $\mu$ g/ml). All panels, \* $P < 0.05$  as compared with time 0 static control.



**Figure 3.** Effects of combined treatment of lipopolysaccharide (LPS) and cellular strain on rat neonatal enterocyte interleukin (IL)-6 and IL-8. Confluent monolayers were incubated with LPS for (a and b) 24 h or (c and d) 48 h in the absence (open bars) or presence (black bars) of cyclic strain. Supra-additive effects of the combined treatment were observed ( $n = 6-12$ , mean  $\pm$  SE). All panels, \* $P < 0.05$  as compared with the time-respective static control.



**Figure 4.** Effects of cellular strain or lipopolysaccharide (LPS) on subconfluent cell growth and proliferation in rat neonatal enterocytes. Cells were seeded at low seeding density; (a) numbers of cells/ $\times 100$  field of view and (b) 5'-bromo-2-deoxyuridine (BrDU)-positive cells were counted ( $n = 6-12$ , mean  $\pm$  SD) (open circle = nonstrained, 0 mg/ml LPS, open triangle = nonstrained, 10 mg/ml LPS, filled circle = strained, 0 mg/ml LPS, filled triangle = strained, 10 mg/ml LPS). All panels, \* $P < 0.05$  as compared with the respective static control.

cells were identified by 5'-Bromo-2-deoxyuridine (BrDU) incorporation (see Methods for details). Cellular strain alone, or in combination with LPS 10  $\mu$ g/ml, caused slower cell growth, with nearly half as many cells as the control by the 48-h time point (Figure 4a). Similarly, the percentage of BrDU positive cells in the cyclic strain treatment groups was approximately one-half of the control or LPS alone at 24 h. Collectively, these data demonstrate that in neonatal enterocytes mechanical strain leads to slower cell growth and division whereas LPS alone has minimal effects under these conditions.

### DISCUSSION

Over the past several decades, improvements in medical care have substantially increased survival and improved outcomes in preterm infants (13,14). Unfortunately the incidence of necrotizing enterocolitis has actually risen slightly over this time period, most likely because of the fact that more premature infants are surviving the initial newborn period (15-17). The advent of NCPAP is one advance that has contributed to improved survival and neonatal outcomes as it can reduce the need or duration of mechanical ventilation, and may lessen the incidence of bronchopulmonary dysplasia (2,3). However, the phenomenon of CPAP belly syndrome, likely due to passive

air-trapping via the esophagus to the stomach and intestine, can cause abdominal distension of a preterm infant, postulated to be the result of minimal sphincter control and abdominal wall muscle mass and tone (6). Some epidemiological studies have concluded that this is a benign manifestation, but other observational studies have noted a potential increase in the rate of necrotizing enterocolitis in premature infants (3,18–22). Although leaders in the area of necrotizing enterocolitis have recently suggested that the pathophysiology involved is multifactorial, there may be subgroups of infants at higher risk of developing NEC due to settings that may allow for a more robust response of the innate immune system of the premature intestine in response to stress (23). To our knowledge, there have been no definitive trials to investigate the impact of NCPAP on the incidence or progression of NEC or any related gastrointestinal ailments in preterm infants. The goal of this study was to test the hypothesis that strain causes cellular stress responses that are already implicated in clinical NEC pathogenesis, either alone or in combination with an additional known stressor, LPS.

These isolated cell studies have shown that the mechanical strain caused by cyclic rhythmic deformation, in addition to exposure to a LPS stimulus causes alterations in the inflammatory cytokine profiles. We chose to study these immature rat intestinal epithelial cells (IEC-6) particularly as they originate from the crypt and this is the area of epithelial intestinal mucosa that would see the most strain during distension as opposed to cells within the villi. In addition, mucosal epithelial cells appear to play a significant role in newborn intestinal diseases associated with local inflammation (23,24). We have demonstrated increased production of both rat IL-6 and growth related oncogene/cytokine induced neutrophil chemoattractant 1 (human IL-8 equivalent) cytokines by the rat intestinal cell line in response to both strain and LPS alone. These cytokines were chosen because they have been shown to be elevated in response to LPS in the cell line by other investigators and have been elevated at 10–20% mechanical strain in other cell types in a dose–response fashion (25–27). Of note, mechanical strain in the absence of an LPS insult caused a significant increase in the production of the pro-inflammatory IL-8 cytokine, likely stimulated by pathways other than through TLR-4. More important, cytokine production in these immature intestinal epithelial cells occurs in an apparent synergistic manner when LPS is added to these cells as opposed to either strain or LPS alone. In addition, many studies have shown that TLR-4 expression is increased in the neonatal intestine with exposure to an LPS stimulus (28,29). Furthermore, other studies have shown that there is increased TLR-4 expression in necrotizing enterocolitis, suggesting that it may be involved in the pathogenesis of the disease (30). Therefore, as we have demonstrated that mechanical strain causes a significant increase in the expression of TLR-4, we speculate this relates to the potential interaction between the inflammatory cytokine pathway and mechanical strain and may contribute to morbidities of the immature intestine.

Another important finding of our study was that mechanical strain significantly decreased immature intestinal cell growth

*in vitro*. Similar to our findings involving cytokine expression, cellular strain was associated with decreased immature enterocyte proliferation. No effect on cell proliferation was seen in the presence of LPS alone. These findings again speak to the concept that the pathogenesis of intestinal problems is multifactorial in nature and may require multiple “hits” to progress to true injury and disease. This finding could significantly impact maturation of the intestinal barrier as well as play a detrimental role in wound healing in repair. It is also interesting to note that presence of cellular strain caused immature enterocytes to shift toward production of inflammatory cytokines, and away from growth and proliferation, seemingly as a “host-defense” response to this mechanical stress. Both of these responses could be considered detrimental to premature infant intestinal maturation. These two responses were not identical to the responses observed with LPS alone (which increased cytokine expression but had no effects on cell growth), and in fact these two stimuli were found in some aspects to be supra-additive. In addition, we observed that strain alone was sufficient to cause an increase in TLR-4 expression by 48 h (Figure 1). Further studies to define the signaling pathways involved in strain-augmented LPS responses, and their contributions to NEC incidence and/or progression are warranted.

It is interesting to note that the temporal profiles observed for the measured cytokines, TLR-4 expression, and cell replication were not identical under the conditions we employed. These disparate profiles suggest that the signaling pathways involved are likely not identical for each of these analytes, and this is consistent with studies using other cell types or conditions that investigate stretch wherein both the nuclear factor kappa  $\beta$  and mitogen activated protein kinases pathways have been implicated. In our experiments it is possible that the earlier upregulation of cytokine release served as an autocrine contributor to later increases in TLR-4 and/or modulator of cell growth and proliferation. Further studies to address these important mechanisms, and particularly to determine signaling interactions of LPS-mediated vs. stretch-mediated effects in this system are clearly warranted. Additional investigations using *in vivo* conditions and intact tissues to address this important clinical problem are also under way.

The pathophysiology of necrotizing enterocolitis is not well understood, which makes the prevention and treatment of this devastating disease very difficult. Because of this uncertainty, it is important to understand the effect these various exposures have on the intestinal epithelium. We postulate that the increased inflammatory cytokines released from the intestinal epithelium in response to mechanical strain, such as that caused by the use of NCPAP, may relate to the pathophysiology of intestinal diseases of the neonate, such as poor motility leading to feeding intolerance and even perhaps necrotizing enterocolitis. The fact that mechanical strain induces the expression of inflammatory cytokines and increases the expression of TLR-4 receptors allows us to speculate that intestinal distension associated with the use of NCPAP, especially in the presence of abnormal gut colonization, results in increased

inflammatory cytokine production and may be a contributing factor to neonatal intestinal morbidities, such as necrotizing enterocolitis and feeding intolerance. It is known that the intestinal epithelium is under continuous and rapid turnover, and in infants (especially preterm infants) it is likely that cellular proliferation is a key component of maintained integrity and rapid maturation. Our data support the concept that intestinal stretch could delay this process, and potentially enhance the risk of more severe conditions such as NEC by predisposing the intestinal tissues to a subsequent challenge (such as LPS). This phenomenon is supported by the general working theory that NEC itself is not a disease of one specific origin but may be an overlay of multiple intestinal insults to a vulnerable patient. Further studies should be conducted and are currently under way to elucidate the relationship between the use of NCPAP and the possible role this modality may play regarding intestinal morbidities. Understanding the interactions between the pathways regulated by these stressors will be imperative for improving the treatment and management of this vulnerable population.

## METHODS

### Isolated Cell Culture

Immature rat intestinal epithelial cells were obtained from American Type Culture Collection (IEC-6, ATCC, Rockville, MD) and were maintained in complete Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, L-alanyl-L-glutamine, insulin, and penicillin/streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. All studies were conducted using cells at less than passage 20.

To investigate the impact of cellular strain on enterocyte responses, we used two experimental conditions. The first was using a confluent monolayer, to investigate the effects of strain on inflammatory cytokine production and *TLR-4* gene expression. The second was using a low seeding density to test the hypothesis that strain affects enterocyte growth and proliferation. When evaluating response to inflammatory stimuli, LPS was added at varying concentrations ranging from 10–150 µg/ml.

### Application of Mechanical Strain

Cells were seeded at a density of either 30,000 or 250,000 cells/cm<sup>2</sup> on type IV collagen-coated Bioflex II 6-well plates (Flexcell International, Hillsborough, NC). Preliminary experiments were conducted to determine conditions providing reliable and stable confluence throughout the study protocols employed. Before application of strain, cells were maintained for 48 h in order to allow them to reach confluence, which was confirmed by light microscopy before use. A 10% mechanical strain was applied using a Flexcell II System (Flexcell International) at a rate of 10 cycles/min. Each cycle consisted of 3 s of membrane deformation followed by 3 s with the membrane in a neutral state. In our preliminary studies, we investigated the relationship between degree of stretch conditions and cellular viability and maintenance of a stable monolayer *in vitro*. We found that the conditions we used herein provided reliable and reproducible cell numbers throughout the study.

### Cytokine Analysis

At each time point with and without an LPS stress, cell culture media was collected for cytokine assays by enzyme-linked immunosorbent assay assays using manufacturer protocols. Commercial enzyme-linked immunosorbent assay kits were used to determine rat IL-6 (BD, Franklin Lakes, NJ) and GRO/CINC-1, the rat ortholog to human IL-8 (R&D Systems, Minneapolis, MN). Each well was analyzed separately and then the average from the wells in each treatment group was obtained. In all cases *n* = 6–9 and intra- and interassay variations were <7%. Cytokine concentrations in cell-free media content were

normalized to numbers of adherent cells at each time point (determined by nuclear cell counts using digital image analysis at the time of media collection).

### Detection of *TLR-4* Gene Expression

RNA was collected using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) per kit instructions. Briefly, cells were lysed directly in wells (after removing media) and lysate was analyzed in triplicate. A total of 600 µl of RLT lysis buffer (Qiagen for all three wells and 30 µl H<sub>2</sub>O was used for elution of RNA for each sample. RNA quality was evaluated on a 1% agarose gel denatured with formaldehyde to ensure that 28 s and 18 s bands were present. Reverse transcriptase real-time PCR was used to detect the presence of *TLR-4* in the samples and was completed using the MuLV Reverse Transcriptase (Applied Biosystems, Carlsbad, CA). Using one-step PCR SYBR green on the iCycler (Bio-Rad Laboratories, Hercules, CA), real-time PCR was performed. The steps included a 3-min denaturation at 95 °C step, followed by a 40-cycle thermal cycling that was composed of a 30-s denaturation at 95 °C, 10-s annealing at 61 °C, and a 30-s extension at 72 °C. The primer used for the *TLR-4* gene was a 160-basepair product with the following sequences: up 5' GGA TTT ATC CAG GTG TGA AA 3' and down 5' TTT GTC TCC ACA GCC ACC A 3' (Integrated DNA Technologies, Coralville, IA). A housekeeping ribosomal protein L30 gene (*RPL30*) was used for normalization of results. This was a 189-basepair product with the following sequences: up 5' GAT CAG ACA AGG CAA AGC GA 3' and down 5' TCA ATG ATA GCC AGT GTG CA 3' (Integrated DNA Technologies).

### Determination of Cell Number and Proliferation

Enterocytes were seeded at 30,000/cm<sup>2</sup> and allowed to adhere for 48 h, then subjected to strain or LPS (10 µg/ml) or their combination. Population growth was determined by staining cells with 4',6-diamidino-2-phenylindole, dihydrochloride and counting numbers of nuclei per field of view at ×100 magnification for each time point with and without LPS stress. In separate wells a pulse of 1 mM 5-BrDU (Sigma-Aldrich, St Louis, MO) for 1 h was used before each timed end point, and BrdU was visualized using anti-BrdU antibody (Millipore, Billerica, MA) and alexafluor 488 secondary antibody (Invitrogen, Carlsbad, CA), according to manufacturers' instructions.

### Data Analysis

Data were analyzed using GraphPad Prism, Version 5.0 (GraphPad Software, La Jolla, CA). Statistical comparisons were made using either *t*-tests or two way ANOVA with Bonferroni corrections where appropriate. A difference was considered significant when the *P* value was <0.05.

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