Toll-like Receptor 4 Is Expressed on Intestinal Stem Cells and Regulates Their Proliferation and Apoptosis via the p53 Up-regulated Modulator of Apoptosis*

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Factors regulating the proliferation and apoptosis of intestinal stem cells (ISCs) remain incompletely understood. Because ISCs exist among microbial ligands, immune receptors such as toll-like receptor 4 (TLR4) could play a role. We now hypothesize that ISCs express TLR4 and that the activation of TLR4 directly on the intestinal stem cells regulates their ability to proliferate or to undergo apoptosis. Using flow cytometry and fluorescent in situ hybridization for the intestinal stem cell marker Lgr5, we demonstrate that TLR4 is expressed on the Lgr5-positive intestinal stem cells. TLR4 activation reduced proliferation and increased apoptosis in ISCs both in vivo and in ISC organoids, a finding not observed in mice lacking TLR4 in the Lgr5-positive ISCs, confirming the in vivo significance of this effect. To define molecular mechanisms involved, TLR4 inhibited ISC proliferation and increased apoptosis via the p53-up-regulated modulator of apoptosis (PUMA), as TLR4 did not affect crypt proliferation or apoptosis in organoids or mice lacking PUMA. In vivo effects of TLR4 on ISCs required TIR-domain-containing adapter-inducing interferon-β (TRIF) but were independent of myeloid-differentiation primary response-88 (MYD88) and TNFα. Physiological relevance was suggested, as TLR4 activation in necrotizing enterocolitis led to reduced proliferation and increased apoptosis of the intestinal crypts in a manner that could be reversed by inhibition of PUMA, both globally or restricted to the intestinal epithelium. These findings illustrate that TLR4 is expressed on ISCs where it regulates their proliferation and apoptosis through activation of PUMA and that TLR4 regulation of ISCs contributes to the pathogenesis of necrotizing enterocolitis.

To maintain intestinal homeostasis, the intestinal epithelium is endowed with a remarkable capacity to undergo self-renewal, a unique property that reflects the activity of a discrete population of progenitor cells located at the base of the intestinal crypts (1–3). The recent identification of precise and reliable markers for intestinal stem cells has allowed for a careful evaluation of their individual capacities to divide and differentiate in both non-stressed states, such as the marker Bmi1 (4–8), as well as under conditions of rapid turnover, such as the marker Lgr5 (9–12). Importantly, the environmental cues that regulate the ability of the progenitor cells of the intestine to divide and the factors that may lead to the loss of intestinal stem cells through apoptosis during intestinal inflammatory diseases remain incompletely examined (13). Given that the intestinal stem cells exist in close association with the microbial flora, it stands to reason that signaling receptors that recognize components of the flora may be present on and have effects on intestinal stem cells. Despite this possibility, the presence of such immune receptors on the intestinal stem cells remains largely unexplored.

The Toll Like Receptors (TLRs) of the innate immune system represent an attractive class of molecules that could serve as receptors on the intestinal stem cells for bacterial products. In this regard, TLR4, which is the receptor for LPS, is known to be expressed within the epithelium of the small intestine where it regulates signaling in response to LPS (14–17). Moreover, exaggerated TLR4 activation has been shown to lead to the development of necrotizing enterocolitis (NEC), a devastating disease of the premature intestine, that is characterized by TLR4-mediated reduction in proliferation and an induction of enterocyte apoptosis (18, 19). These findings raise the intriguing possibility that TLR4 itself may be expressed on the intestinal stem cells and thus regulate their function. We now hypothe-

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‡ The abbreviations used are: TLR, Toll-like receptor; NEC, necrotizing enterocolitis; PUMA, p53-up-regulated modulator of apoptosis; qRT-PCR, quantitative RT-PCR; PCNA, proliferating cell nuclear antigen.

Significance: This is the first study showing that ISC regulation by microbial receptors contributes to NEC pathogenesis.
esize that the intestinal stem cells themselves express TLR4 and that the activation of TLR4 directly on the intestinal stem cells has a direct role on the ability of intestinal stem cells to either proliferate or to undergo apoptosis. In support of this hypothesis, using a variety of knockout and transgenic strains, we now show that TLR4 is expressed on intestinal stem cells and that the activation of TLR4 leads to a loss of proliferation and increase in apoptosis in a mechanism that is dependent upon the activation of the p53-up-regulated modulator of apoptosis (PUMA) in the pathogenesis of NEC.

**EXPERIMENTAL PROCEDURES**

**Cells, Materials, and Reagents**—The small intestinal cell line IEC-6 was obtained from the ATCC and cultured as described previously (20). To generate IEC-6 cells that were stably deficient in PUMA, IEC-6 cells were transduced with lentiviral particles (Invitrogen) containing PUMA-shRNA (Open Biosystems, Huntsville, AL) using the four plasmid lentiviral packaging system in permissive HEK 293 cells. Stable integration of lentivirus was obtained by selection of cells using media containing puromycin (5 μg/ml), and knockdown of PUMA was verified by qRT-PCR and Western blot analysis. TLR4-deficient IEC-6 cells were generated as described (21). Primary intestinal cultures (organoids) were isolated and maintained in culture on Matrigel according to the methods of Sato et al. (12). LPS (Escherichia coli 0111:B4 purified by gel filtration chromatography, > 99% pure) was obtained from Sigma-Aldrich. Antibodies were obtained as follows: GFP (Millipore), PUMA (Abcam), Cleaved caspase 3 (Cell Signaling Technology), BrdU (Novus), PCNA (Santa Cruz), TLR4 (Imgenix), and E-cadherin (Invitrogen).

**Mice**—PUMA−/− mice were from The Jackson Laboratory (JAX). TLR4−/− mice were generated in our laboratory by first generating a flox×TLR4−/− mouse that was then bred with the EIIa-Cre mouse (22) (Jackson Labs) to generate TLR4−/− mice as described (23). C57Bl/6, Lgr5-EGFP-IRES-creERT2, Gt(Rosa)26Sor1(ACTB-tdTomato, EGFP)luc reporter (mT/mG), and PUMA−/− mice (24) were from Jackson Laboratories. Mice lacking TLR4 in Lgr5-positive intestinal stem cells were generated by first crossing the Lgr5-EGFP-IRES-creERT2 mice (12) with mT/mG. The offspring of this cross were then bred with TLR4loxP/loxP mice to create an inducible system whereby TLR4 could be removed from Lgr5-positive cells upon injection of tamoxifen. Mice globally lacking TNFα, TNFR1, MyD88, and TRIF were obtained from Jackson Labs.

**Induction of Endotoxemia and Necrotizing Enterocolitis**—All experiments were approved by the Children’s Hospital of Pittsburgh Animal Care Committee and the Institutional Review Board of the University of Pittsburgh. Tamoxifen was given by intraperitoneal injection (Sigma-Aldrich) (25) of 5 mg/ml per day prior to the experimental model to achieve deletion of TLR4 from the Lgr5-positive cells within the intestinal crypts. Endotoxemia was induced by intraperitoneal injection of LPS (5 μg/kg) 18 h prior to sacrifice. Experimental NEC was induced in 10-day-old mice as described previously (21) using formula gavage (Similac Advance infant formula (Abbott Nutrition); Esbilac canine milk replacer, 2:1) five times/day and hypoxia (5%O2, 95%N2) for 10 min in a hypoxic chamber (Billups-Rothenberg) twice daily for 4 days. The severity of disease was determined on histologic sections of the terminal ileum by a pediatric pathologist who was blinded to the study condition according to our previously published scoring system from 0 (normal) to 3 (severe) (25). Sections of the terminal ileum were harvested at the end of the model and processed for RNA, protein, and immunopathology analysis (16).

To delete PUMA specifically from the intestine, neonatal mice were gavage-fed lentiviruses expressing PUMA shRNA at postnatal day 7 a total of 100 μl of virus (10^4 PFU/ml) once daily for 7 days. Littermate controls received a lentivirus containing scrambled shRNA as a negative control.

Intestinal samples were obtained from human neonates undergoing intestinal resection for NEC or at the time of stoma closure (“healed NEC”). All human tissue was obtained and processed as discarded tissue via a waiver of consent with approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue procurement guidelines.

**Quantitative Real-time Polymerase Chain Reaction**—Quantitative real-time PCR was performed as described previously using the Bio-Rad CFX96 real-time system (Bio-Rad) (18) using the primers listed below. The expression of the following genes by qRT-PCR was measured relative to the housekeeping genes β-actin, GAPDH, or Ribosomal Protein L15 (RPLO) using the primers included in the supplemental data. Where indicated, crypt cells were isolated from Lgr5-Rosa-mT/mG-TLR4−/− mosaic mice 24 h after tamoxifen injections and verified for viability using LiveDead Aqua (Invitrogen) reagents according to the instructions of the manufacturer. Aqua-negative (viable) cells were then selectively sorted into GFP-positive (TLR4-negative) and GFP-negative (TLR4-positive) populations using FACS Aria (BD Biosciences). Flow-sorted GFP-positive and GFP-negative cells were processed for total RNA isolation using the RNeasy kit (Qiagen) followed by cDNA synthesis and quantitative RT-PCR using gene-specific primers. Settings were chosen to select for TLR4 positivity and not the low background level Lgr5-GFP signal.

**Fluorescent in Situ Hybridization (FISH)—**FISH was performed on paraffin-imbedded tissues from C57Bl/6 and TLR4−/− mice using the Digoxigenin (DIG) system from Roche Applied Sciences according to the protocol of the manufacturer (Roche Diagnostic Corporation, Indianapolis, IN). Briefly, DIG-labeled TLR4 (forward, CAGCAGAAAGTCCTGATGACA and reverse, ATTTCCCTGAAAAGCTTGGT) and Lgr5 (forward, TTGGAGAAAGGAGAGCTGGA and reverse, AGTCATGGGGTAAGCTGTG) RNA probes were synthesized from mouse cDNA generated by RT-PCR and cloning into the pGEM-T easy vector system (Promega). FISH was performed using 100 ng/ml of each probe for a total hybridization time of 16 h at 42 °C using 50% formamide in the hybridization mixture. Counterstaining was performed prior to mounting with maminobenzidine substrate (Vector Laboratories) for 20 min at room temperature, and then sections were imaged using an upright Imager.Z1 microscope with AxioCam MRc5 (Carl Zeiss).

**Determination of Enterocyte Proliferation and Apoptosis**—Enterocyte proliferation in vitro was measured using the color-
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imetric XTT (2,3-bis(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay (Sigma-Aldrich)). 5000 cells were plated to 50% confluence without serum, and the extent of proliferation was expressed as a percent of the maximum proliferation rate. For assessment of in vivo proliferation, sections of terminal ileum were immunostained with antibodies to PCNA or Ki67 or subjected to RT-PCR for PCNA as described (23). In parallel, mice were injected with the nucleotide analog BrdU (Invitrogen, 10 μl/g of total body weight) 2–18 h prior to sacrifice and immunostained with anti-BrdU antibodies.

To measure apoptosis, cells or tissue were immunostained with antibodies against cleaved caspase 3 or TUNEL using the ApoTag in situ apoptosis detection kit (Millipore) according to the protocol of the manufacturer (21).

Statistical Analysis—All experiments were repeated at least in triplicate, with more than 100 cells/high-power field. For endotoxiaemia, at least three mice/group were assessed. For NEC, over 10 mouse pups per group were included, and litter-matched controls were included in all cases. Statistical analysis was performed using SPSS 13.0 software. Analysis of variance was used for comparisons for experiments involving more than two experimental groups. Two-tailed Student’s t test was used for comparison for experiments consisting of two experimental groups. For analysis of the severity of NEC, χ-square analysis was performed. In all cases, statistical significance was accepted at p < 0.05 between groups.

RESULTS

TLR4 Is Expressed in the Intestinal Crypts and on Lgr5-positive Intestinal Stem Cells and Regulates the Apoptosis and Proliferation of Intestinal Stem Cells—To determine whether TLR4 could play a direct role in the extent of proliferation or apoptosis of intestinal stem cells, we first sought to define whether TLR4 was expressed in the proliferative region of the small intestine, i.e., the intestinal crypts. To do so, we performed FISH using specific probes for TLR4 mRNA in the small intestine of wild-type and TLR4-deficient mice. As shown in Fig. 1A, TLR4 mRNA could be detected largely within the intestinal crypts, where its detection appeared to be most apparent in the most inferior portion of the crypts where the Lgr5-positive crypt-based columnar cells were detected by FISH (Fig. 1, C–D), as shown previously (1). No mRNA was detected in TLR4-deficient mice, confirming the specificity of the FISH probe for TLR4 (Fig. 1B). These findings raised the possibility that TLR4 may be expressed on the intestinal stem cells themselves. To evaluate this possibility directly, we adopted the methods of Clevers and colleagues (12) to harvest the intestinal stem cells using mechanical separation and differential centrifugation from mice that express GFP driven by the Lgr5-promoter (Lgr5-EGFP-IRES-creERT2 mice), and then performed flow cytometry using anti-GFP antibodies to detect the Lgr5-positive cells. As shown in Fig. 1E, we identified a population of GFP-positive (i.e., Lgr5-positive) cells (quadrants II and IV), and approximately 50% of these cells routinely expressed TLR4 (quadrant II). To investigate the effects of TLR4 activation on intestinal stem cell proliferation and apoptosis, we next isolated crypt organoids from both wild-type and TLR4-deficient mice and maintained these crypts in culture on Matrigel in the presence or absence of LPS. As shown in Fig. 1F, the addition of LPS caused a marked increase in apoptosis, as measured by increased cleaved caspases 3 staining within the cultured crypt organoids (Fig. 1, H–I), and a significant reduction in the expression of PCNA (G), consistent with a reduction in proliferation. Importantly, the addition of LPS had no effect on either proliferation or apoptosis in crypts that were harvested from TLR4-deficient mice, confirming the specificity of the effect of LPS for TLR4 (Fig. 1, F and G, J and K). TLR4 activation with LPS also caused a significant impairment of the growth and organization at 48 h of the organoids that were obtained from wild-type mice (Fig. 1, N and O) as compared with either untreated organoids (L and M) or organoids obtained from TLR4-deficient mice under identical conditions (P–S). Taken together, these findings illustrate that TLR4 activation on the intestinal stem cells can increase apoptosis and reduce proliferation in vitro.

TLR4 Activation on Intestinal Stem Cells Increases Apoptosis and Reduces Proliferation in Vivo—To determine the physiological consequences of the activation of TLR4 on intestinal stem cells, we next injected either wild-type or TLR4-deficient mice with LPS or saline and evaluated the effects on crypt proliferation and apoptosis in vivo. As shown in Fig. 2, the injection of wild-type mice with LPS markedly increased the apoptosis within the crypts, as manifest by increased expression of cleaved caspases 3 (Fig. 2, A and B), and significantly reduced proliferation within crypts, as determined by reduced uptake of the nucleotide analog BrdU (E and F). These findings were not observed in TLR4-deficient mice (Fig. 2, C and D, G and H), which confirmed that the effects of LPS signaling on intestinal crypts required TLR4 expression (M and N). However, these studies did not exclude the possibility that LPS could be inducing apoptosis of intestinal stem cells via an intermediate such as TNFα, which has been shown to mediate the effects of LPS on apoptosis in other cells (26). To exclude this possibility directly, we injected mice that were deficient in either TNFα or in the TNFα receptor (TNFR1) with either saline or LPS and assessed the extent of apoptosis within the intestinal crypts by TUNEL immunostaining. As shown in Fig. 2, I–L and O, neither the inhibition of TNFα nor the inhibition of the TNFα receptor prevented the induction of apoptosis in response to LPS injection. These results exclude the possibility that TNFα could be mediating the effects of LPS on intestinal stem cell apoptosis and suggest that a direct, TLR4-mediated signaling pathway is involved.

Selective Deletion of TLR4 from the Lgr5+ Intestinal Stem Cells Prevents the Effects of LPS on Intestinal Stem Cell Apoptosis and Proliferation—The above studies raise the possibility that TLR4 signaling reduces crypt proliferation and increases crypt apoptosis by acting on some cell type other than the Lgr5+/TLR4+ intestinal stem cells identified in Fig. 1, which could then secondarily impair crypt proliferation and apoptosis. To exclude this possibility directly and to determine precisely the effects, if any, of TLR4 signaling in the Lgr5+ intestinal stem cells, we generated mice in which TLR4 was selectively deleted from Lgr5+ intestinal stem cells. To do so, we first generated a reporter mouse strain by breeding the Lgr5-EGFP-IRES-creERT2 mice to a mouse expressing loxp-
flanked membrane-Tomato/membrane-Green (mT/mG). This reporter mouse strain was then bred with TLR4 \( \text{lox}^P/\text{lox}^P \) mice to generate an inducible system whereby the administration of tamoxifen could result in the simultaneous deletion of TLR4 and activation of green fluorescence (Lgr5-Rosa-mT/mG-\( \text{lox}^P/\text{lox}^P \)), thereby revealing both the fact that TLR4 had been deleted as well as the precise cells from which the deletion occurred by the identification of green cells. Because of the variegated expression of the Lgr5-Egfp-IRES-creERT2 transgene in the parent mice, the resulting Lgr5-Rosa-mT/mG-\( \text{lox}^P/\text{lox}^P \) mice progeny are mosaic, with some crypts expressing TLR4 (and thus not staining green), and some that do not.

**FIGURE 1.** TLR4 is expressed in the intestinal crypts and on Lgr5-positive cells and regulates the apoptosis and proliferation of intestinal stem cells. A–D, confocal micrographs showing fluorescent in situ hybridization for TLR4 (green, A and B) and Lgr5 (C and D) in wild-type and TLR4 \( \text{lox}^P/\text{lox}^P \) mice as indicated. The hashed line indicates the zoomed region as indicated. Tissue sections were costained for DAPI (blue). Scale bar = 100 \( \mu \)m. E, flow cytometry profile of intestinal stem cells isolated from Lgr5-EGFP-IRES-creERT2 mice that were labeled with antibodies against GFP (Lgr5) and TLR4. Quadrant II shows cells that are both TLR4-positive and Lgr5-positive. F and G, quantification of apoptosis and proliferation in the crypt organoids pertaining to H–K. *, \( p < 0.05 \) versus saline; **, \( p < 0.05 \) versus LPS. Ctrl, control. H–K, confocal micrographs of intestinal crypts from wild-type and TLR4 \( \text{lox}^P/\text{lox}^P \) mice as indicated in the absence or presence of LPS and immunostained with antibodies to cleaved caspases 3 (red) and DAPI (blue). Scale bar = 100 \( \mu \)m. L–S, bright field micrographs of intestinal crypts harvested from either wild-type or TLR4 \( \text{lox}^P/\text{lox}^P \) mice and grown in Matrigel for 48 h with or without LPS (10 \( \mu \)g/ml) as indicated. Scale bar = 100 \( \mu \)m.
express TLR4 (and thus staining green). This novel system allows for paired comparisons between TLR4-deficient (i.e., stained green because of the Rosa-mT/mG-GFP transgene) and crypts that are TLR4-expressing (i.e., no green staining) within the same mice under the same conditions.

To confirm that we can reliably detect the Lgr5-positive progeny, we first bred the Lgr5-Egfp-IRES-creERT2 with Rosa26mT/mG, in which the GFP expression identified the presence of the Lgr5/H11001 cells and their progeny within the crypts (at 12 h of tamoxifen injection, Fig. 3A–H) and the epithelial cells that are derived from these crypts (at 48 h of tamoxifen injection, B) and thus documented the reliability of the Lgr5 promoter driving this reporter system.

To assess the expression of TLR4 and Lgr5 in the TLR4/H11001 (GFP-) and TLR4- (GFP+/H11001) cells, crypts were isolated and sorted for the GFP expression as described under “Experimental Procedures” and analyzed by RT-PCR for TLR4 and Lgr5. As shown in Fig. 3, G and H, the TLR4+/H11001 intestinal stem cells showed a 50-fold increase in TLR4 expression compared with the TLR4- cells, whereas both crypt cell populations expressed Lgr5 at comparably high levels.

The effects of TLR4 activation in the TLR4-expressing intestinal stem cells are shown in Fig. 3, D–F, I and J, after mice were injected with either saline or LPS. Specifically, the injection of LPS caused an increase in crypt cell apoptosis, as revealed by an increase in staining of cleaved caspases 3 (Fig. 3, C and D) and a significant reduction in proliferation as revealed by a reduction in expression of Ki67 (Fig. 3, E–F) that was only in crypt cells that express TLR4 (i.e., did not stain green). Importantly, TLR4-negative (i.e., green) crypts were unaffected by LPS injection and thus had no change in either their proliferation or apoptosis (Fig. 3, C–F, see quantification in I and J). Taken together, these data confirm that TLR4 signaling within the intestinal crypts on Lgr5/H11001 cells intestinal stem cells leads to impaired crypt dynamics, as manifested by increased apoptosis and reduced proliferation. We next sought to determine the molecular mechanisms involved in this effect.

**PUMA Mediates the Increased Apoptosis and Reduced Proliferation within Intestinal Crypts That Are Induced by TLR4**—To identify the molecular mechanisms by which TLR4 activation could mediate the increased apoptosis and reduced proliferation in intestinal crypts, we turned our attention to the 23-kDa protein PUMA (p53 up-regulated modulator of apoptosis), a member of the Bcl-2 superfamily that inhibits the promitotic protein p53 to prevent cell division and initiate a proapoptotic program (27–29). To directly interrogate the role of PUMA, we

![TLR4 activation on intestinal stem cells increases apoptosis and reduced proliferation in vivo.](image-url)
first studied the duodenal crypt-derived cell line IEC-6, which normally expresses PUMA and which we engineered to be either deficient in PUMA using lentiviral transduction of PUMA shRNA (TLR4-kd), or to overexpress PUMA using adenoviral expression of PUMA (TLR4-over, Fig. 4A). As shown in Fig. 4B, the treatment of wild-type IEC-6 cells with LPS led to an increase in the expression of PUMA, which was not seen in TLR4-kd cells (B) and which correlated with an increase in apoptosis as measured by the expression of cleaved caspases 3 (Fig. 4, C, G, and H). As shown in Fig. 4D, overexpression of PUMA reduced IEC-6 proliferation, whereas knockdown of PUMA increased proliferation, confirming the role of PUMA on proliferation in this system. The injection of LPS into wild-type mice also caused a marked increase in the expression of PUMA within the crypts, providing some physiological relevance of this effect (Fig. 4, E and F). Importantly, knockdown of PUMA in IEC-6 cells prevented the LPS-induced increase in apoptosis (Fig. 4, C, I, and J) and significantly reduced the impairment in cell proliferation (not shown), whereas cells that overexpress PUMA showed such a dramatic increase in apoptosis that further changes after LPS exposure were not reliably quantifiable (not shown).

To further evaluate the role of PUMA on TLR4-mediated proliferation and apoptosis of crypt cells, we next harvested intestinal crypts from wild-type and PUMA knockout mice and exposed these crypts to LPS. As shown in Fig. 4, treatment of the crypt organoids with LPS caused a marked increase in apoptosis (Fig. 4, K and L; saline 5 ± 2% versus LPS, 17 ± 4%, p < 0.05) and a reduction in proliferation as determined by fold change of PCNA expression relative to GAPDH by qRT-PCR (saline, 1 versus LPS 0.2 ± 2, p < 0.05) in wild-type crypt organoids that was not seen in PUMA-deficient organoids (apoptosis, Fig. 4, M and N; saline, 5 ± 2% versus LPS, 5 ± 4%, NS and proliferation PCNA RT-PCR, saline 1 versus LPS 1.1 ± 2, NS).

To determine whether PUMA could regulate crypt proliferation and apoptosis in response to TLR4 activation in vivo, both wild-type and PUMA-deficient mice were next injected with either saline or LPS, and the extent of proliferation and apoptosis in the crypts was assessed. Although LPS injection into wild-type mice caused a significant increase in apoptosis as manifest by increased TUNEL staining (Fig. 4, O and P; % TUNEL-positive crypts, saline 3 ± 2% versus LPS 15 ± 4%, p < 0.05) and a reduction in proliferation as seen by reduced PCNA staining (S and T; % PCNA-positive crypts, saline 55 ± 2% versus LPS 5 ± 4%, p < 0.05), the injection of LPS into PUMA knockout mice showed no effects on apoptosis (Q and R, TUNEL, saline 5 ± 2% versus LPS 5 ± 4%, NS; p < 0.05) or proliferation (U and V, saline 75 ± 2% versus LPS 85 ± 4%, not significant (NS)). It is noteworthy that PUMA−/− mice show an induction in serum IL-6 after injection with LPS to levels similar to that of wild-type mice (wild-type, 45 ± 2 pg/ml; PUMA−/−, 42 ± 2 pg/ml, NS), excluding the possibility that the PUMA-deficient strain is globally unresponsive to LPS. Taken together, these data demonstrate that PUMA regulates TLR4-mediated apoptosis and proliferation in intestinal crypts both in vitro and in vivo. We next sought to determine the factors downstream of TLR4 that could mediate the effects on PUMA induction.

The LPS-mediated Induction of PUMA in Intestinal Crypts Requires TRIF but not MyD88—TLR4 signaling is known to occur through either MyD88-dependent pathways, which result in the activation of NFκB, and MyD88-independent pathways, which involve TRIF and lead to the activation of IRF-3 and the production of interferon β (30). To assess
whether the induction of PUMA in response to TLR4 activation occurred in a MyD88-dependent or MyD88-independent manner, we next injected MyD88−/− and TRIF−/− mice with either saline or LPS and assessed the degree of expression of PUMA at both the mRNA and protein levels. As shown in Fig. 5A, LPS caused a significant induction in PUMA mRNA in MyD88−/−
mice that was not seen in TRIF<sup>−/−</sup> mice, indicating a TRIF-dependent role for PUMA induction in response to LPS. This finding was confirmed at the protein level, as LPS injection led to an increase in PUMA expression by both SDS-PAGE (Fig. 5B, i and ii) and immunoconfocal microscopy in MyD88<sup>−/−</sup> mice (C and D) that was not observed in TRIF<sup>−/−</sup> mice (E and F). Taken together, these findings indicate that LPS causes an increase in PUMA in a TRIF-dependent and MyD88-independent manner. We next sought to evaluate the physiological relevance of these findings in the pathogenesis of necrotizing enterocolitis.

The Deletion of PUMA Protects Mice from the Development of Necrotizing Enterocolitis and Reverses the Deleterious Effects on Crypt Apoptosis and Proliferation—NEC is a devastating disease of premature infants that causes acute inflammation and necrosis of the small intestine that we and others have shown to be caused by elevated mucosal TLR4 signaling (18, 19, 23). We and others have also found that NEC is associated with a reduction in enterocyte proliferation and an increase in enterocyte apoptosis, although the mechanisms involved remain incompletely understood (23, 31, 32). We now demonstrate that wild-type mice that were subjected to an experimental model of NEC, which involves 4 days of intermittent hypoxia followed by serial formula gavage, showed an increase in the expression of PUMA within the intestinal crypts (Fig. 6, A and B) that was not observed in TLR4-deficient mice (C and D), confirming that the induction of NEC leads to a TLR4-dependent increase in PUMA expression. To evaluate the role of PUMA in the induction in crypt apoptosis and reduction in proliferation observed in NEC, we subjected both wild-type and PUMA-deficient mice to experimental NEC and observed that in contrast to wild-type mice, PUMA-deficient mice displayed normal intestinal crypts without evidence of either apoptosis (Fig. 6, E–H and Q) or reduced proliferation (I–L and R), an overall improvement in the architecture of the mucosa (M–P and S), and a reduction in the expression of proinflammatory inducible nitric oxide synthetase (iNOS) (T). PUMA-deficient mice also displayed effects on the expression of the apoptosis-regulatory genes Bax and Bad but not Bcl2 in response to LPS, as determined by RT-PCR (Fig. 6, U–W). It is noteworthy that in sections that were obtained from the intestine of human infants with NEC, we identified an increase in crypt apoptosis that was not seen in healthy control infants (Fig. 6, X and Y) and that correlated with an increase in the expression of PUMA within the intestine of humans with NEC (Fig. 4Z).

In the final series of experiments, we sought to determine whether PUMA signaling in the intestinal crypts themselves or in some other cell type was responsible for the induction of apoptosis and inhibition of proliferation in response to TLR4 activation. To do so, we selectively inhibited PUMA up-regu-
Figure 6. The deletion of PUMA protects mice from the development of necrotizing enterocolitis and reversed the deleterious effects on crypt apoptosis and proliferation. A–D, representative confocal micrographs of terminal ileal crypts from wild-type and TLR4−/− mice that were either breast-fed (control) or subjected to experimental NEC and immunostained for PUMA (green) and DAPI (blue). Scale bar = 100 μm. E–L, representative confocal micrographs showing crypts from the terminal ileum from wild-type and PUMA−/− mice that were either breast-fed (control) or subjected to experimental NEC and immunostained with DAPI (blue), cleaved caspases 3 (green, arrows), or PCNA (red) as indicated. Scale bar = 100 μm. Arrows show apoptotic cells in the crypt base. M–P, representative H&E-stained sections from terminal ileum of newborn wild-type and PUMA−/− mice that were either allowed to breast-feed or subjected to NEC as indicated. Q–S, quantification of apoptosis (Q) pertaining to E–H; proliferation (R) pertaining to I–L; NEC severity (S) pertaining to M–P. *, p < 0.05 versus breast-fed control; **, p < 0.05 versus NEC wild-type. T, qRT-PCR showing expression of iNOS in the intestinal mucosa of wild-type and PUMA−/− mice that were breast-fed (control) or subjected to experimental NEC. *, p < 0.05 versus breast-fed control; **, p < 0.05 versus NEC wild-type. U–W, qRT-PCR showing expression of Bcl2 (U), Bax (V), and Bad (W) in the intestinal mucosa of wild-type or PUMA−/− mice that were injected with either saline or LPS. *, p < 0.05 versus saline wild-type; **, p < 0.05 versus saline PUMA−/−. X–Z, representative confocal micrograph of human ileum stained for TUNEL (green, arrows) and DAPI (blue) in infants with NEC (Y) and in control infants (X). Scale bar = 100 μm. Z, SDS-PAGE showing PUMA (top) and β-actin (bottom) in human intestinal mucosa of a premature infant without and with NEC as indicated. Ctrl, control.
tion in experimental NEC in vivo through the oral administration of a lentiviral-expressed PUMA shRNA (Fig. 7, A–C). SDS-PAGE of mucosal scrapings from mice administered lentiviral shRNA PUMA or control virus showing PUMA knockdown is shown in J. The induction of NEC resulted in an increase in the expression of PUMA in the intestinal crypts in wild-type mice (Fig. 7, A–C) and in mice administered scrambled shRNA by lentivirus (not shown), which was not observed in mice that had been administered the PUMA shRNA lentivirus (C). Importantly, the inhibition of PUMA in the intestinal epithelium restored intestinal stem cell proliferation (Fig. 7K), maintained the mucosal architecture (G–I), and attenuated NEC severity (L), whereas the administration of scrambled shRNA had no effect (not shown). Taken together, these findings illustrate the importance of PUMA-mediated effects on apoptosis and proliferation in the intestinal crypts in response to TLR4 activation in the pathogenesis of necrotizing enterocolitis.

DISCUSSION

We now show that the Lgr5-positive intestinal stem cells within the crypts of the small intestine in the newborn mouse express TLR4, that activation of TLR4 within the intestinal crypts results in reduced proliferation and increased apoptosis, that TRIF is required for the effects of TLR4 on crypt apoptosis, and that these events require the activation of PUMA in response to TLR4. In seeking to define the physiological relevance of these findings, we further show that TLR4-induced PUMA activation within the intestinal crypts of the newborn mice plays a key role in the development of NEC, a devastating disease of premature newborns that is characterized by exaggerated TLR4 signaling (19, 33). It remains a possibility that TLR4 activation within the intestine leads to the apoptosis of villi in addition to the crypts within the newborn intestine, with the former leaving the host vulnerable to bacterial translocation and the latter render-
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...ing the host inadequately able to heal the intestinal defects because of a marked impairment in crypt cell proliferation. The current studies are the first, therefore, to define that the development of NEC may reflect in part a reduction in crypt progenitor cells because of exaggerated TLR4 signaling in this compartment, and thus challenge current dogmas in the field that generally views the development of NEC as a nonspecific exaggerated response to endogenous pathogens in the premature host (34). These findings raise the possibility that strategies that enhance mucosal healing through effects on the now identified TLR4-PUMA axis may be harnessed therapeutically for this devastating disorder.

Although the current findings show that TLR4 plays a direct role in the regulation of crypt apoptosis and proliferation and exclude the possibility that TNFα may exert a role as an intermediate molecule, the question remains as to what physiological role, if any, TLR4 may play in the crypts themselves. Although this work is the first instance of TLR4 expression on the intestinal stem cell (ISC), the question remains as to what biological role, if any, TLR4 may play in the crypts themselves. Although this work is the first instance of TLR4 expression on the intestinal stem cell (ISC), the question remains as to what biological role, if any, TLR4 may play in the crypts themselves.

In related findings, Ragab and colleagues (37) recently determined that Rho/AKT signaling can restrict the innate immune response in intestinal stem cells in Drosophila, providing additional links between immune responses and intestinal proliferation. It is tempting to now speculate that TLR4 may play a parallel role in the mammalian host by regulating the apoptotic burst or by modifying the JAK-STAT and JNK pathways (36). In related findings, Ragab and colleagues (37) recently determined that Rho/AKT signaling can restrict the innate immune response in intestinal stem cells in Drosophila, providing additional links between immune responses and intestinal proliferation. It is tempting to now speculate that TLR4 may play a parallel role in the mammalian host by regulating the apoptotic burst or by modifying the JAK-STAT and JNK pathways (36).

In summary, we have now identified a novel paradigm whereby TLR4 activation on intestinal stem cells induces a program of increased apoptosis and reduced proliferation. These findings have significant impact on the pathogenesis of necrotizing enterocolitis, in which TLR4 signaling is exaggerated. Further studies to define the precise role of TLR4 on the intestinal stem cells, the effects of TLR4 signaling under baseline conditions, and the precise conditions in which TLR4 signaling within the intestinal stem cells leads to a PUMA–induced stem cell arrest will no doubt provide essential new information in the factors that regulate homeostasis within the newborn small intestine and in the pathogenesis of necrotizing enterocolitis.

REFERENCES
1. Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Hageebarth, A., Korfving, J., Begthel, H., Peters, P. J., and Clevvers, H. (2012) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449, 1003–1007
2. Neal, M. D., Richardson, W. M., Sodhi, C. P., Russo, A., and Hackam, D. J. (2011) Intestinal stem cells and their roles during mucosal injury and repair. J. Surg. Res. 167, 1–8
3. Garrison, A. P., Helmrath, M. A., and Dekaney, C. M. (2009) Intestinal stem cells. J. Pediatr. Gastroenterol. Nutr. 49, 2–7
4. Sangiorgi, E., and Capecci, M. R. (2008) Bmi1 is expressed in vivo in intestinal stem cells. Nat. Genet. 40, 915–920
5. Yan, K. S., Chia, L. A., Li, X., Ootani, A., Su, J., Lee, J. Y., Su, N., Luo, Y., Heilshorn, S. C., Amieva, M. R., Sangiorgi, E., Capecci, M. R., and Kuo, C. J. (2012) The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. Proc. Natl. Acad. Sci. U.S.A. 109, 466–471
6. Yu, T., Chen, X., Zhang, W., Colon, D., Shi, J., Napier, D., Rychahou, P., Lu, W., Lee, E. Y., Weiss, H. L., Evers, B. M., and Liu, C. (2012) Regulation of the potential marker for intestinal cells, Bmi1, by β-catenin and the zinc finger protein KLF4. Implications for colon cancer. J. Biol. Chem. 287, 3760–3768
7. Dehmer, J. J., Garrison, A. P., Speck, K. E., Dekaney, C. M., Van Landeghem, L., Sun, X., Henning, S. J., and Helmrath, M. A. (2011) Expansion of intestinal epithelial stem cells during murine development. PLoS ONE 6, e27070
8. Tian, H., Blies, B., Warming, S., Leong, K. G., Rangell, L., Klein, O. D., and de Sauvage, F. J. (2011) A reserve stem cell population in small intestine renders Lgr5–positive cells dispensable. Nature 478, 255–259
9. Carmon, K. S., Lin, Q., Gong, X., Thomas, A., and Liu, Q. (2012) LGR5...
Interacts and cointernalizes with Wnt receptors to modulate Wnt/β-catenin signaling. *Med. Cell Biol.* 32, 2054–2064

10. Kim, T. H., Escudero, S., and Shivdasani, R. A. (2012) Intact function of Lgr5 receptor-expressing intestinal stem cells in the absence of Paneth cells. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3932–3937

11. Schepers, A. G., Vries, R., van den Born, M., van de Wetering, M., and Cleurs, H. (2011) Lgr5 intestinal stem cells have high telomerase activity and randomly segregate their chromosomes. *EMBO J.* 30, 1104–1109

12. Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., van Es, J. H., Abo, A., Kuja, P., Peters, P. J., and Clevers, H. (2009) Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche. *Nature* 459, 262–265

13. Dekaney, C. M., Gulati, A. S., Garrison, A. P., Helmrah, M. A., and Hening, S. J. (2009) Regeneration of intestinal stem/progenitor cells following doxorubicin treatment of mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 297, G461–G470

14. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freundgen, M., Riccardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice. Mutations in Tlr4 gene. *Science* 282, 2085–2088

15. Neal, M. D., Leaphart, C., Levy, R., Prince, J., Billiar, T. R., Watkins, S., Li, J., Cetin, S., Ford, H., Schreiber, A., and Hackam, D. J. (2006) Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. *J. Immunol.* 176, 3070–3079

16. Afrazi, A., Soda, C. P., Good, M., Jia, H., Siggers, R., Yazji, I., Ma, C., Neal, M. D., Prindle, T., Grant, Z. S., Branca, M. F., Ozojek, J., Chang, E. B., and Hackam, D. J. (2012) Intracellular heat shock protein-70 negatively regulates TLR4 signaling in the newborn intestinal epithelium. *J. Immunol.* 188, 4543–4557

17. Good, M., Siggers, R. H., Soda, C. P., Afrazi, A., Alkhudari, F., Egan, C. E., Neal, M. D., Yazji, I., Jia, H., Lin, J., Branca, M. F., Ma, C., Prindle, T., Grant, Z., Shah, S., Slagle, D., Zed, Paredes, J., Ozojek, J., Gittes, G. K., and Hackam, D. J. (2012) Amniotic fluid inhibits Toll-like receptor 4 signaling in the fetal and neonatal intestinal epithelium. *Proc. Natl. Acad. Sci. U.S.A.* 109, 11330–11335

18. Leaphart, C. L., Cavolo, J., Gribar, S. C., Cetin, S., Li, J., Branca, M. F., Dubowski, T. D., Soda, C. P., and Hackam, D. J. (2007) A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J. Immunol.* 179, 4808–4820

19. Jilling, T., Simon, D., Lu, J., Meng, F. J., Li, D., Schy, R., Thomson, R. B., Soliman, A., Arditi, M., and Caplan, M. S. (2006) The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. *J. Immunol.* 177, 3273–3282

20. Qureshi, F. G., Leaphart, C., Cetin, S., Li, J., Grishin, A., Watkins, S., Ford, H. R., and Hackam, D. J. (2005) Increased expression and function of integrins in enterocytes by endotoxin impairs epithelial restitution. *Gastroenterology* 128, 1012–1022

21. Richardson, W. M., Soda, C. P., Russo, A., Siggers, R. H., Afrazi, A., Gribar, S. C., Neal, M. D., Dai, S., Prindle, T. Jr., Branca, M., Ma, C., Ozojek, J., and Hackam, D. J. (2010) Nucleotide-binding oligomerization Domain-2 Inhibits Toll Like Receptor-4 Signaling in the Intestinal Epithelium. *Gastroenterology* 139, 904–917

22. Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W., and Westphal, H. (1996) Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5860–5865

23. Soda, C. P., Shi, X. H., Richardson, W. M., Grant, Z. S., Shapiro, R. A., Prindle, T. Jr., Branca, M., Russo, A., Gribar, S. C., Ma, C., and Hackam, D. J. (2010) Toll-like receptor-4 inhibits enterocyte proliferation via impaired β-catenin signaling in necrotizing enterocolitis. *Gastroenterology* 138, 185–196

24. Villunger, A., Michalak, E. M., Coutlas, L., Müllauer, F., Böck, G., Ausserlechner, M. J., Adams, J. M., and Strasser, A. (2003) p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 302, 1036–1038

25. Gribar, S. C., Soda, C. P., Richardson, W. M., Anand, R. J., Gittes, G. K., Branca, M. F., Jakub, A., Shi, X. H., Shah, S., Ozojek, J. A., and Hackam, D. J. (2009) Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. *J. Immunol.* 182, 636–646

26. Ruemmele, F. M., Beaulieu, J. F., Dionne, S., Levy, E., Seidman, E. G., Cerf-Bensussan, N., and Lentze, M. J. (2002) Lipopolysaccharide modulation of normal enterocyte turnover by toll-like receptors is mediated by endogenously produced tumour necrosis factor α. *Gut* 51, 842–848

27. Qiu, W., Carson-Walter, E. B., Kuan, S. F., Zhang, L., and Yu, J. (2009) PUMA suppresses intestinal tumorigenesis in mice. *Cancer Res.* 69, 4999–5006

28. Qiu, W., Carson-W ALTER, E. B., Liu, H., Epperly, M., Greenberger, J. S., Zambetti, G. P., Zhang, L., and Yu, J. (2007) p53 independent induction of PUMA mediates intestinal apoptosis in response to ischaemia-reperfusion. *Gut* 56, 645–654

29. Abraham, C., and Medzhitov, R. (2011) Interactions between the host innate immune system and microbes in inflammatory bowel disease. *Gastroenterology* 140, 1729–1737

30. Khailova, L., Mount, Patrick, S. K., Arganbright, K. M., Halpern, M. D., Castagnoli, P., Layton, B., and Beutler, B. (1998) Defective LPS signaling in the newborn intestinal epithelium. *J. Immunol.* 160, 4543–4557

31. Khailova, L., Mount, Patrick, S. K., Arganbright, K. M., Halpern, M. D., Castagnoli, P., Layton, B., and Beutler, B. (1998) Defective LPS signaling in the newborn intestinal epithelium. *J. Immunol.* 160, 4543–4557
44. Suzuki, A., Sekiya, S., Gunshima, E., Fujii, S., and Taniguchi, H. (2010) EGF signaling activates proliferation and blocks apoptosis of mouse and human intestinal stem/progenitor cells in long-term monolayer cell culture. Lab. Invest. 90, 1425–1436
45. Potten, C. S., Owen, G., Hewitt, D., Chadwick, C. A., Hendry, H., Lord, B. I., and Woolford, L. B. (1995) Stimulation and inhibition of proliferation in the small intestinal crypts of the mouse after in vivo administration of growth factors. Gut 36, 864–873
46. Dirisina, R., Katzman, R. B., Goretsky, T., Managlia, E., Mittal, N., Williams, D. B., Qiu, W., Yu, J., Chandel, N. S., Zhang, L., and Barrett, T. A. (2011) p53 and PUMA independently regulate apoptosis of intestinal epithelial cells in patients and mice with colitis. Gastroenterology 141, 1036–1045
47. Qiu, W., Wu, B., Wang, X., Buchanan, M. E., Regueiro, M. D., Hartman, D. J., Schoen, R. E., Yu, J., and Zhang, L. (2011) PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. J. Clin. Invest. 121, 1722–1732