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Toll-like-receptor-4 Inhibits Enterocyte Proliferation via Impaired β -catenin Signaling in Necrotizing Enterocolitis

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Abstract

Background and Aims—Necrotizing enterocolitis (NEC), the leading cause of gastrointestinal death from gastrointestinal disease in preterm infants, is characterized by exaggerated TLR4 signaling and decreased enterocyte proliferation through unknown mechanisms. Given the importance of β -catenin in regulating proliferation of many cell types, we hypothesize that TLR4 impairs enterocyte proliferation in NEC via impaired β -catenin signaling.

Methods—Enterocyte proliferation was detected in IEC-6 cells or in ileum or colon from wild-type, TLR4-mutant or TLR4^{-/-} mice after induction of NEC or endotoxemia. β -catenin signaling was assessed by cell fractionation or immunofluorescence microscopy to detect its nuclear translocation. Activation and inhibition of β -catenin were achieved via cDNA or siRNA respectively. TLR4 in the intestinal mucosa was inhibited using adenoviruses expressing dominant-negative TLR4.

Results—TLR4 activation significantly impaired enterocyte proliferation in the ileum but not colon in newborn but not adult mice, and in IEC-6 enterocytes. β -catenin activation reversed these effects in vitro. To determine the mechanisms involved, TLR4 activation phosphorylated the upstream inhibitory kinase GSK3 β , causing β -catenin degradation. NEC in both mouse and humans was associated with decreased β -catenin and increased mucosal GSK3 β expression. Strikingly, the inhibition of enterocyte β -catenin signaling in NEC could be reversed, and enterocyte proliferation restored, through adenoviral-mediated inhibition of TLR4 signaling in the intestinal mucosa.

Conclusion—We now report a novel pathway linking TLR4 with inhibition of β -catenin signaling via GSK3 β activation, leading to reduced enterocyte proliferation in vitro and in vivo. These data provide additional insights into the pathogenesis of diseases of intestinal inflammation like NEC.

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Xia-hua Shi – no conflicts exist; Chhinder P. Sodhi – no conflicts exist; Ward M. Richardson – no conflicts exist; Steven C. Gribar – no conflicts exist; Thomas Prindle Jr – no conflicts exist; Maria Branca – no conflicts exist; Anthony Russo – no conflicts exist; Congrong Ma – no conflicts exist; Richard Shapiro – no conflicts exist; Zachary Grant – no conflicts exist; David J. Hackam – no conflicts exist.

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INTRODUCTION

Necrotizing enterocolitis (NEC) is the leading cause of death from gastrointestinal disease in preterm infants, and remains a major cause of long term disability in those who survive the disease^{1, 2}. Despite the increasing frequency with which NEC is diagnosed, its pathogenesis remains largely uncertain, although there is general acceptance that its development reflects persistent intestinal mucosal injury³. In seeking to understand how mucosal injury in NEC occurs, we have recently shown that NEC development requires activation of the innate immune receptor Toll like receptor 4 (TLR4) on enterocytes, and that TLR4 activation by bacterial endotoxin (lipopolysaccharide, LPS) inhibits enterocyte proliferation and leads to disease progression^{4, 5}. Importantly, the mechanisms by which TLR4 activation leads to an inhibition in enterocyte proliferation in the pathogenesis of NEC remain largely unknown. To investigate this further, we now focus on the canonical β -catenin signaling pathway, a series of protein-protein interactions with an established role in the regulation of enterocyte proliferation via the upstream inhibitor GSK3 β . Although a link between TLR4 and β -catenin remains to be established, such a link could provide a novel explanation for the initiation and propagation of the mucosal injury seen in NEC.

We now report the novel finding that TLR4 activation inhibits enterocyte proliferation in vitro and in vivo via impaired β -catenin signaling after activation of the AKT-GSK3 β signaling pathway. These effects were restricted to the small intestine of newborn mice, suggesting relevance in the development of NEC. Strikingly, the TLR4-mediated inhibition of β -catenin signaling could be partially restored in mice with NEC through adenoviral-mediated inhibition of TLR4 signaling in the intestinal mucosa. The current findings support a novel linkage between TLR4 and inhibitory β -catenin signaling in enterocytes, suggest an important physiological context where such signaling may be important, and provide additional insights into the development of intestinal mucosal injury in the pathogenesis of NEC.

METHODS

Cell culture, mice and reagents

IEC-enterocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained as described⁷. Immortalized primary murine embryonic fibroblasts (MEF) were isolated from 13 day old TLR4-wild-type and TLR4-mutant embryos and cultured in DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine and 10 μ g/ml blasticidin (InvivoGen, San Diego, CA). Lipopolysaccharide (LPS, 99% pure) was purified from *E. coli* O127:B8 by gel filtration chromatography (Sigma-Aldrich, Saint Louis, MO). In all cases, the concentration of LPS used was based upon the measured concentration of LPS found in the stools of mice with experimental NEC as we recently reported (50 μ g/ml)⁸. Antibodies were as follows: β -catenin, tubulin, and histone H1, pCNA from Santa Cruz (Santa Cruz, CA); phospho-GSK-3 β and phospho-AKT from Cell Signaling (Danvers, MA); phospho- β -catenin from Sigma. LiCl from Sigma. SiRNA to GSK3 β , β -catenin and TLR4 from Dharmacon (Lafayette, CO, 100 nM final concentration), and were used as described⁸. TLR4^{-/-} mice were generated by first generating a floxp-TLR4 mice, in collaboration with Ozgene (Bentley, Australia), which we then bred with the EIIa-Cre mouse (Jackson Labs) to generate the global TLR4^{-/-} mouse. These mice were genetically TLR4 deficient, and failed to mount a septic response to the intraperitoneal injection of LPS.

Plasmids expressing constitutively active β -catenin were generously provided by Dr. S. P. Monga, University of Pittsburgh⁹ and were transfected into IEC-6 cells using Lipofectamine 2000.

Details of RT-PCR and primers are included in Online Supplementary Material.

Determination of enterocyte proliferation

Enterocyte proliferation in vitro was measured using the colorimetric XTT(2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide assay (Sigma-Aldrich). 5000 cells were plated to 50% confluence without serum and the extent of proliferation was expressed as a percent of maximum proliferation rate i.e. the rate of untreated cells. Wild-type, TLR4-mutant or TLR4^{-/-} mice were injected with either saline or LPS (5 mg/kg) for 3h. The terminal ileum was then freshly harvested, fixed in formalin and immunostained with antibodies to the proliferation marker pCNA as described¹⁰, or subjected to analysis by RT-PCR using mouse specific primers. To quantify pCNA staining in the intestinal crypts, images of pCNA were digitized, regions of interest were drawn around the crypts, and the extent of pCNA expression was evaluated using ImageJ software(NIH).

Generation of adenoviruses bearing wild-type and mutant TLR4

Replication-deficient recombinant adenoviruses that express GFP-tagged mouse wild-type or dominant negative TLR4-cDNA and control adenovirus expressing GFP, were prepared with the Adeno-X-Expression System2 kit (Clontech), and verified for their ability to inhibit TLR4 signaling in vivo as we recently described⁴.

Immunohistochemistry, cell fractionation and SDS-PAGE

Immunostaining of IEC-6, mouse/human intestine was performed as described^{11, 12}. Nuclear and cytoplasmic fragments in IEC-6 cells were separated according to the methods of Mei et al¹³, and were probed with antibodies against β -catenin, α -tubulin (cytoplasm) and histone-H1 (nucleus). SDS-PAGE was performed as described⁵.

Induction of experimental NEC and endotoxemia

All experiments were approved by the Children's Hospital of Pittsburgh Animal Care Committee and the Institutional Review Board of the University of Pittsburgh. C3H/HeOJ (i.e. TLR4 wild-type) and C3H/HeJ (i.e. TLR4 mutant) strains were obtained from Jackson Laboratories (Bar Harbor, ME). Endotoxemia was induced in 2 week old mice by the intraperitoneal injection of LPS (*Escherichia coli* 0111:B4 purified by gel-filtration chromatography, >99% pure, 5 mg/kg, Sigma-Aldrich, St. Louis, MO). 2h later, animals were euthanized, and samples of the terminal ileum were obtained 1cm proximal to the ileocecal valve. Experimental NEC was induced in 10–14 day old mice as we described¹² using formula gavage (Similac Advanced infant formula (Ross Pediatrics):Esbilac canine milk replacer 2:1) five times/day, and hypoxia (5% O₂, 95% N₂) for 10min in a hypoxic chamber (Billups-Rothenberg, DelMar, CA) twice daily for 4d. Where indicated, mice were enterally administered adenoviral GFP, GFP-wild-type TLR4, or GFP-dominant-negative TLR4 twice daily for three days (240uL, 10¹² PFU). The expression of GFP in mucosal scrapings of the intestine, lung and liver were assessed by RT-PCR for GFP; intestines were studied for GFP expression by confocal microscopy, and confirmed to be expressed in the intestinal mucosa with no detectable expression in the liver or lung⁴. Intestinal samples were obtained from human neonates undergoing intestinal resection for NEC, for unrelated indications (control), or at time of stoma closure were processed as we described⁴.

Statistical Analysis

Data presented are mean \pm SEM, and comparisons are by two-tailed Student's t test or ANOVA, with statistical significance accepted for p<0.05. All experiments were repeated in at least triplicate, with at least five mice per group where indicated.

RESULTS

TLR4 activation inhibits enterocyte proliferation in vitro and in vivo

We first sought to assess the effects of TLR4 activation with lipopolysaccharide (LPS) on enterocyte proliferation in vitro and in vivo. As shown in Figure 1, LPS caused a dose-dependent reduction in the extent of enterocyte proliferation, as determined by colorimetric XTT assay (**panel A**) and reduced expression of the cell proliferation marker pCNA by SDS-PAGE (**panels B**) and confocal microscopy (note the loss of green pCNA staining with increased concentration of LPS in **panel C**). There was no effect of LPS on viability as assessed by Trypan blue exclusion (not shown), and LPS caused a dose-dependent increase in NF κ B activation and IL-6 expression (Supplemental Figure 1). We next sought to confirm a specific role for TLR4 activation in inhibition of enterocyte proliferation by LPS. Knockdown of TLR4 with TLR4-specific siRNA - which resulted in a >90% reduction in TLR4 expression compared to scrambled siRNA-transfected cells (**panel D upper**) - reversed the inhibitory effects of LPS on enterocyte proliferation (**panel D lower**). Importantly, LPS also significantly reduced proliferation in murine embryonic fibroblasts obtained from TLR4 wild-type mice but not TLR4-mutant mice, suggesting that the inhibitory effects of TLR4 are not confined to IEC-6 cells (**Panel E**). Taken together, these findings strongly indicate that TLR4 activation with LPS significantly inhibits enterocyte proliferation in vitro.

To define whether TLR4 activation would inhibit enterocyte proliferation in vivo, TLR4 wild-type (C3H/HeOJ) and TLR4-mutant (C3H/HeJ) mice were injected with either saline or LPS, and the extent of enterocyte proliferation was measured within the enterocytes of the terminal ileum. As shown in Figure 2, LPS significantly reduced enterocyte proliferation in wild-type mice as compared to saline injected animals, yet had no effect in TLR4-mutant mice as shown by immunostaining (**panels A–B**) and RT-PCR for the proliferation marker pCNA in the intestinal mucosa (**panel D**). The inhibitory effects of LPS on enterocyte proliferation in vivo were further demonstrated by SDS-PAGE showing a reduction in pCNA expression compared to saline injected animals (**panel C**). As shown in **panel E**, LPS did not decrease the rate of enterocyte proliferation in the ileum of adult mice (checkered bars) or in the colon of newborn mice (grey bars), suggesting that the effect may be specific for the small intestine of newborns. We next sought to determine the molecular mechanisms involved.

TLR4 activation inhibits enterocyte proliferation via the canonical beta-catenin signaling pathway

Given the established role of the canonical β -catenin signaling pathway in the regulation of the proliferation of enterocytes and other cells, we next sought to determine whether β -catenin signaling may mediate the inhibitory effects of TLR4 signaling on enterocyte proliferation. Signaling via the canonical β -catenin pathway results in the translocation of β -catenin from the cytoplasm to the nucleus, which results in the transcription of a variety of genes that induce cell proliferation¹⁴. As shown in Figure 3 (A i–iii and B), under control conditions, β -catenin could be detected in IEC-6 cells in both nuclear and cytosolic locations. By contrast, treatment with LPS led to a re-distribution of β -catenin into a predominantly cytoplasmic location, suggesting an inhibition of nuclear translocation in response to LPS (Figure 3A iv–vi, B). LPS also caused a dose-dependent decrease in β -catenin expression in nuclear as compared with cytosolic fractions purified from IEC-6 cells (Figure 3 **panel C**). To determine directly whether TLR4 activation may inhibit enterocyte proliferation via effects on β -catenin signaling, three specific siRNA's against β -catenin - individually and in combination (“pooled”) - were transfected in IEC-6 enterocytes, and the pooled siRNA resulted in a greater than 95% reduction in β -catenin expression as compared with control siRNA (Figure 3D i–ii). Inhibition of β -catenin prevented further decreases in enterocyte proliferation after the subsequent addition of LPS (Figure 3D iii). Strikingly, overexpression of active β -catenin reversed the inhibitory

effects of LPS on enterocyte proliferation as measured by expression of pCNA (Figure 3D iv). Taken together, these findings demonstrate that TLR4 inhibits enterocyte proliferation via effects on the pathways that regulate β -catenin signaling.

A novel link between TLR4 and AKT-GSK3 β signaling mediates the inhibitory effects of LPS on enterocyte proliferation

It has been previously demonstrated that the ability of β -catenin to translocate into the nucleus is dependent upon its stability within the cytoplasm, which is governed by the activity of the upstream serine/threonine kinase glycogen-synthase 3-beta (GSK3 β). GSK-3 β negatively regulates β -catenin by phosphorylation of Ser33/37/Thr41 - leading to its degradation - while AKT, a critical upstream negative regulator of GSK3 β , inactivates GSK-3 β by phosphorylation of its Ser9 residue¹⁵. We therefore next tested whether the TLR4-induced β -catenin-mediated inhibition of enterocyte proliferation required GSK3 β . As shown in Figure 4 **panel A**, LPS dose-dependently increased the phosphorylation of β -catenin, which was associated with reduced total β -catenin expression, consistent with its proteosomal degradation. LPS also caused a dose-(Figure 4, **panel B**) and time- (Figure 4, **panel C**) dependent *decrease* in the phosphorylation of GSK3 β in enterocytes, a finding consistent with an expected *increase* in GSK3 β kinase activity as demonstrated by the increased phosphorylation of β -catenin. Since phosphorylation of GSK3 β is known to be mediated by AKT¹⁶, we next assessed the effects of LPS on AKT phosphorylation and expression. As shown in Figure 4 **panels B-C**, LPS caused a dose- and time-dependent decrease in phosphorylation of AKT and a decrease in overall AKT expression (consistent with the observed decrease in phosphorylation of GSK3 β over time). Inhibition of p-AKT with LY290043 prevented the LPS-induced reduction in phosphorylation of GSK3 β , confirming the link between AKT and GSK3 β signaling (Figure 4 **panel D, schematic panel F**). In support of the physiological relevance of these findings, LPS caused an increase in the expression of phospho- β -catenin and a corresponding increase in the expression of GSK3 β in TLR4-expressing mice that were injected with LPS Figure 4 **panel E** (Quantification and statistical analysis of all blots is given in Supplementary Figure 2).

To test directly therefore whether GSK3 β signaling mediates the inhibitory effects of LPS on enterocyte proliferation, IEC-6 cells were treated with lithium chloride, a remarkably specific GSK3 β inhibitor¹⁷, which significantly reversed the LPS-induced inhibition of nuclear translocation of β -catenin in IEC-6 cells (Figure 5 **panels A-B**), and also reversed the LPS-mediated inhibition in enterocyte proliferation (Figure 5 **panel C**). To confirm this finding, siRNA knockdown of GSK3 β completely reversed the inhibitory effects of LPS on enterocyte proliferation (Figure 5 **panels D**). Taken together, we conclude that TLR4-mediated inhibition in enterocyte proliferation is mediated via GSK3 β signaling and correspondingly decreased β -catenin activity.

NEC is associated with TLR4-mediated induction of β -catenin-GSK3 β signaling in enterocytes and an inhibition in enterocyte proliferation in mice; similar changes occur in human infants with NEC

We next sought to determine the physiological relevance of these findings in the pathogenesis of NEC, a disease that we have shown to be characterized by TLR4-mediated reduced enterocyte proliferation⁸. As is shown in Figure 6 **panels A-C**, NEC in TLR4-expressing mice was associated with a striking reduction in β -catenin and increase in GSK3 β within terminal ileal enterocytes as demonstrated by microscopy and RT-PCR compared to breast-fed controls; these changes were not observed in TLR4-mutant mice. Of interest, exposure of mice to formula feeding alone led to a 50% reduction in enterocyte proliferation as measured by intestinal mucosal RT-PCR and β -catenin compared to breast fed animals before evidence of

histological injury was observed, suggesting that the reduction in enterocyte proliferation may be an early event contributing to NEC pathogenesis ($p < 0.05$).

To determine whether these effects were seen in other strains, we generated TLR4^{-/-} mice and injected them with LPS (1mg/kg), which significantly decreased β -catenin and pCNA expression and increased GSK3 β ; these changes were not seen in TLR4^{-/-} mice (Figure 6 **panel D**). Finally, in human infants with NEC, there was a dramatic decrease in β -catenin and pCNA and increase in GSK3 β expression compared with the terminal ileum from infants without NEC (Figure 6 **panel E**). We note that the control infants were on average 6 to 8 weeks older than the infants with NEC, corresponding to the time at which stoma reversal was performed. Taken together, these findings provide a strong link between TLR4 and β -catenin-GSK3 β signaling in enterocytes in the pathogenesis of NEC, and provide direct physiological relevance of the novel pathway identified in Figure 3–Figure 5.

Inhibition of enterocyte TLR4 signaling in mice with NEC restores enterocyte proliferation and reverses the changes in β -catenin and GSK3 β expression

We next sought to determine whether we could reverse the inhibitory effects of TLR4 activation on enterocyte proliferation by blocking TLR4 signaling, and therefore reverse the inhibition in enterocyte proliferation in mice with NEC. We have recently demonstrated that GFP-tagged dominant negative TLR4 bearing the P710H mutation that occurs in the C3H/HeJ mouse inhibits LPS-induced TLR4 signaling in enterocytes and protects against experimental NEC⁴, and that enteral administration of adenoviral GFP, GFP-wild-type TLR4 and GFP-dominant negative TLR4 lead to expression that is restricted to the enterocytes⁴. As shown in Figure 7, mice with NEC that were enterally administered adenoviral-GFP-TLR4 or -GFP demonstrated reduced enterocyte proliferation as manifest by decreased pCNA (**panel A**), decreased β -catenin (**panel B**) and increased GSK3 β (**panel C**) compared with breast fed controls, consistent with the findings shown in Figure 6, and providing further evidence for a link between TLR4-mediated β -catenin-GSK3 β signaling and enterocyte proliferation in vivo. Strikingly, the administration of adenoviral-dominant-negative TLR4 significantly reversed the expression of β -catenin and pCNA, and reduced expression of the inhibitory molecule GSK3 β , to levels observed in breast fed mice (**grey bars in Figure 7 panels A–C**). As an important control, in TLR4 mutant mice, adding back wild-type-TLR4 decreased enterocyte proliferation, compared to TLR4 mutant mice that were administered GFP or dominant negative TLR4 (Figure 7 **panel D**). The distribution of the GFP-tagged proteins were each localized predominantly to the enterocytes (**Panel D i–iv**). These findings indicate a critical role for TLR4 activation in the inhibition of enterocyte proliferation in vivo in the pathogenesis of NEC, in part via effects on the β -catenin-GSK3 β signaling pathway.

DISCUSSION

We now describe the identification of a novel and potentially important link between TLR4 signaling and activation of the AKT-GSK3 β pathway leading to the inhibition of enterocyte proliferation, an effect that plays a role in the pathogenesis of NEC. The current findings are consistent with previous work indicating that enterocyte proliferation may be inhibited after endotoxin exposure^{18, 19}, yet extend these observations by providing both mechanistic insights and physiological relevance. In support of the relevance of the current findings to the pathogenesis of NEC, we now show that TLR4 activation – which we and others have previously shown to be important in NEC pathogenesis^{8, 20} – leads to decreased β -catenin and increased GSK3 β expression in the intestinal mucosa in both human and murine NEC, while inhibition of TLR4 signaling in enterocytes in vivo reverses these effects and restores levels of enterocyte proliferation in experimental NEC. It is interesting to note that other diseases of intestinal inflammation are associated with impaired enterocyte proliferation, including various

models of colitis that mimic inflammatory bowel disease in humans^{21, 22}. Given the observation that TLR4 expression has been shown to be increased in specimens obtained from regions of active ulcerative colitis in humans²³, it is tempting to speculate that by inhibition of enterocyte proliferation – perhaps via effects on β -catenin signaling - TLR4 activation could contribute to persistent mucosal injury in these inflammatory intestinal diseases also.

With further respect to NEC pathogenesis, we note that it has recently been reported that in the newborn intestine, vaginal delivery and LPS exposure lead to LPS unresponsiveness by the primary enterocytes²⁴. Based upon our recent discovery that the hypoxia and endotoxemia that characterize NEC leads to persistent upregulation of intestinal TLR4⁸, we now propose that part of the mechanism of NEC reflects the inability of the intestine to down-regulate TLR4 signaling in order to become tolerant to the luminal bacteria. This would be expected to lead to exaggerated TLR4 signaling upon bacterial colonization, resulting in impaired enterocyte proliferation and persistent susceptibility to mucosal injury as seen in NEC. These findings expand the current understanding of the role of TLR4 in intestinal homeostasis, while also providing novel insights into the molecular mechanisms that lead to NEC.

How can the current findings be reconciled with the observation that patients with chronic intestinal inflammation have an increased propensity to develop intestinal tumors, suggesting that enterocyte proliferation may be increased at least transiently during states of chronic intestinal injury? First, we point out that the current findings may be specific for the newborn small intestine, as TLR4 activation did not decrease enterocyte proliferation in either adult intestine or newborn colon. Second, it is well established that downstream mediators of TLR4 signaling – including the transcription factor NF κ B and associated molecules - have established roles in the development of tumors, in part via transcription of oncogenes²⁵, which could lead to induction of tumors after chronic intestinal inflammation. And third, while we now demonstrate that TLR4 signaling in enterocytes is responsible for the inhibition of enterocyte proliferation *in vivo*, it is quite possible that in conditions of colitis that favor the development of tumors, the effects of TLR4 signaling may occur via hematopoietic cells and not on enterocytes. In support of this possibility, Abreu et al has recently used TLR4 wild-type and mutant chimeric mice to show that TLR4 signaling in leukocytes plays a key role in colitis associated tumor formation²⁶. We now propose that in the intestinal inflammatory environment, TLR4 activation in different cell types leads to disparate effects: in newborn enterocytes, an inhibition of cell turnover is observed leading to mucosal injury, while in the setting of chronic NF κ B activation – potentially via effects on leukocytes - tumorigenesis may occur. It is also likely that the extent and duration of inflammation, the age of the individual and the location of the inflammation (colon vs. ileum) play important roles in determining whether enterocyte proliferation is impaired, as we now show, as opposed to the more long term models of colitis in which tumors may form²⁷.

In summary, we now provide evidence for a novel link between TLR4 and the GSK3 β - β -catenin signaling pathway in enterocytes, and show that the interaction of these signaling pathways plays a key role in the pathogenesis of NEC via impaired enterocyte proliferation. The mechanisms by which this pathway is regulated *in vivo* may open up future directions of study regarding both the pathogenesis and potential treatment of devastating diseases such as NEC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Role in the study:

Shi – study concept and design, data acquisition, data analysis; Sodhi – study concept and design, data acquisition, data analysis; Steve Gribar – data acquisition; Ward Richardson – data acquisition, Zachary Grant – data acquisition; Richard A. Shapiro – data acquisition; Thomas Prindle – data acquisition; Maria Branca – data acquisition; Anthony Russo – data Acquisition; David Hackam – study concept and design, data analysis, supervision, obtaining funding, drafting of manuscript.

Abbreviations

| | |
|--------------|---------------------------|
| GSK3 β | glycogen-synthase 3-beta |
| TLR4 | Toll like receptor-4 |
| NEC | necrotizing enterocolitis |

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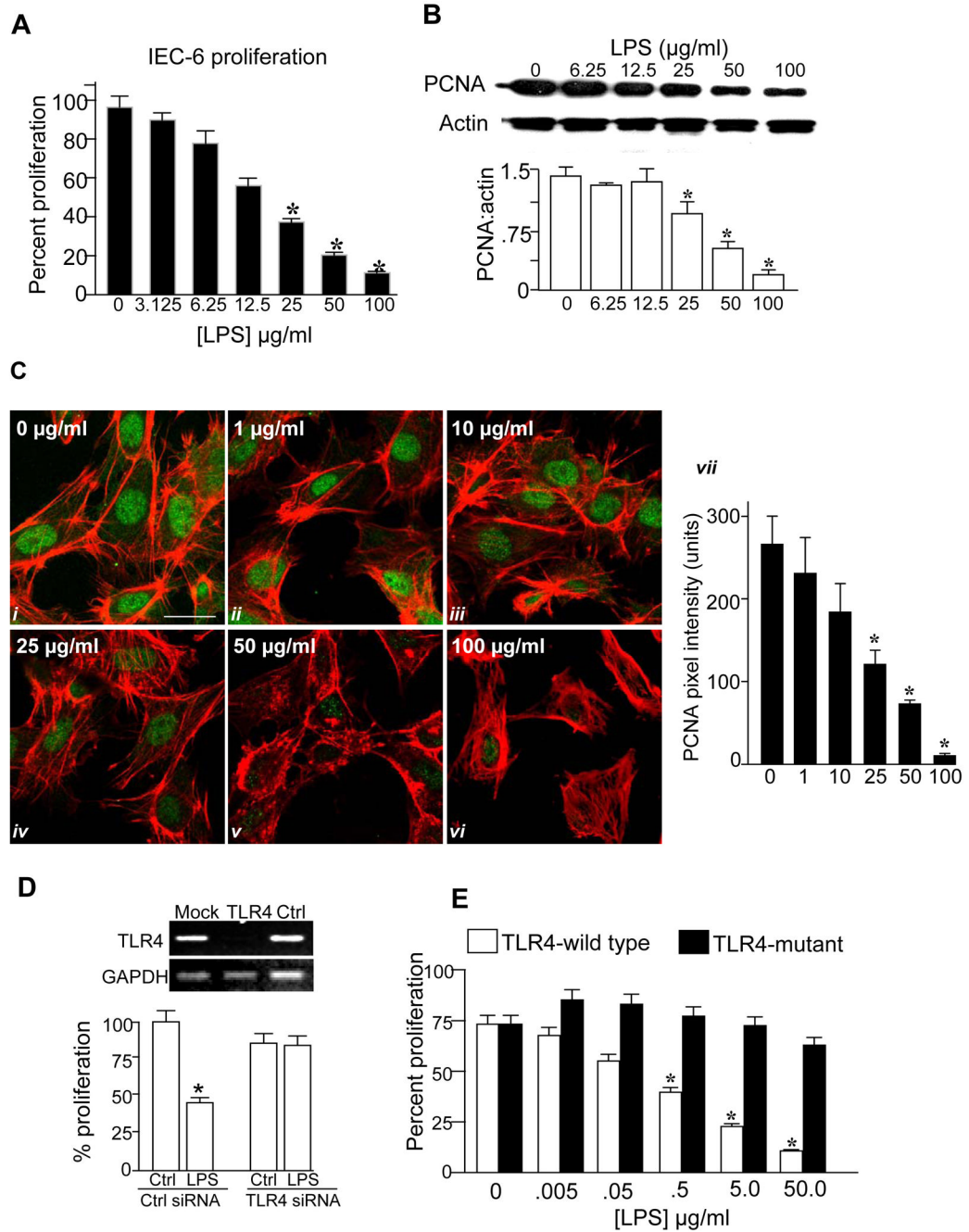


Figure 1. TLR4 activation inhibits enterocyte proliferation in vitro

A: Percent proliferation in serum-starved IEC-6 cells treated with LPS (3h) at the indicated dosage as determined by XTT assay. **B:** SDS-PAGE showing pCNA and actin expression and their quantified expression ratio, in IEC-6 cells treated with LPS. * $p < 0.01$ by ANOVA. **C:** Confocal micrographs showing expression of pCNA (green) and actin (red) treated with LPS for 3h at the indicated concentration; quantification of pCNA pixel density is shown, size bar=10 μm . $p < 0.05$ vs. untreated cells, ANOVA. **D:** RT-PCR showing expression of TLR4 and GAPDH in IEC-6 cells that were either untreated (“mock”), or treated with siRNA to TLR4 (“TLR4”) or to no known targets (“ctrl”), and percent proliferation in IEC-6 cells treated as indicated. **E:** Effect of inhibition of TLR4 by siRNA on proliferation in embryonic fibroblasts

from TLR4-wild-type (C3H/HeOJ) or TLR4-mutant (C3H/HeJ) mice treated with LPS for 16h as indicated. $p < 0.05$ vs. untreated cells, ANOVA

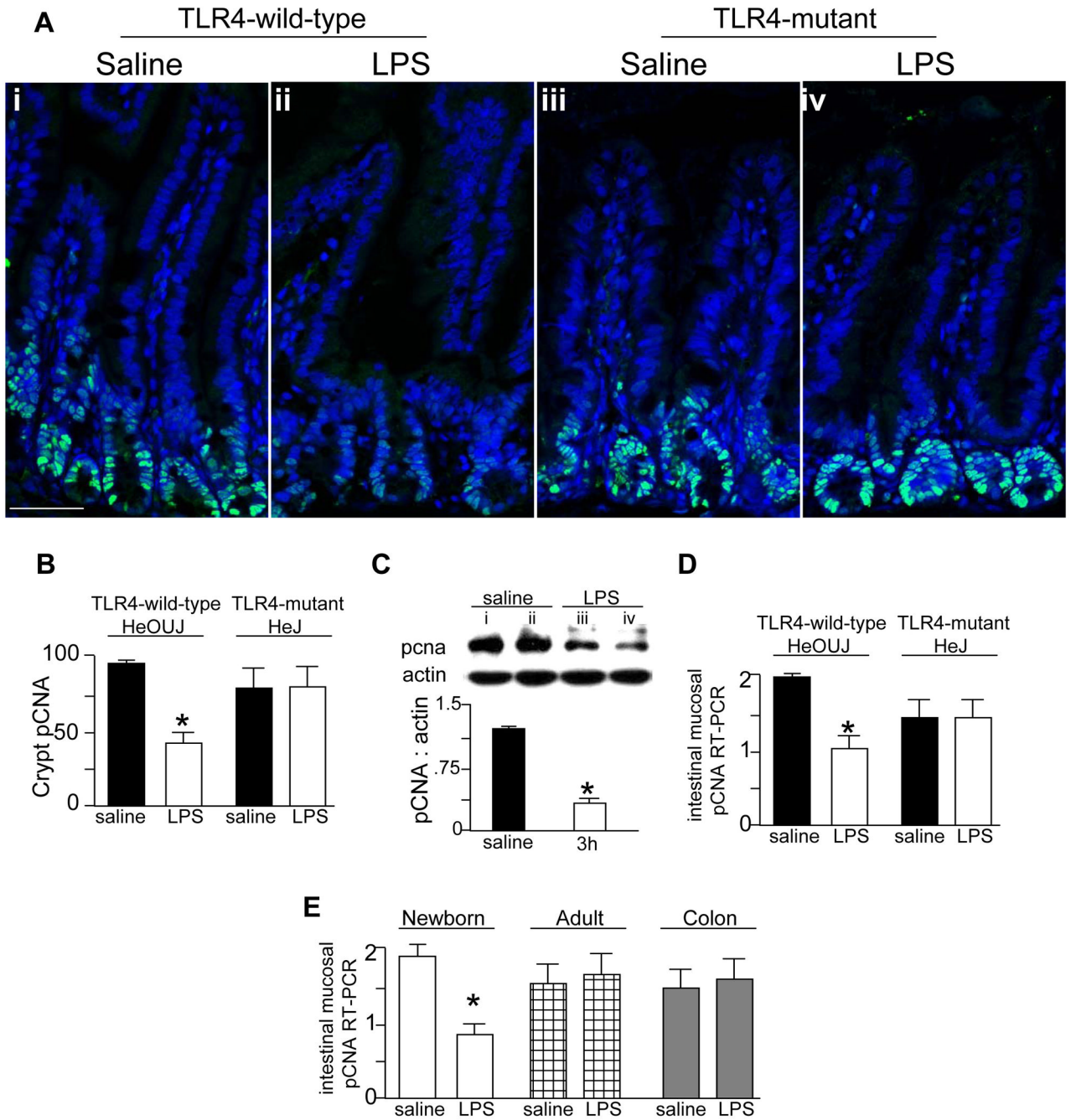


Figure 2. TLR4 activation leads to an inhibition of enterocyte proliferation in vivo

A: Confocal micrographs showing sections of terminal ileum from TLR4-wild-type (**i–ii**) and TLR4-mutant mice (**iii–iv**) that were injected with LPS or saline and immunostained with pCNA. Size bar = 250 μ m. **B:** Quantification of pCNA staining in crypts in **A**, * $p < 0.01$ vs. saline C3H/HeOUJ by ANOVA. Representative of 3 separate experiments with at least 5 mice per group. **C:** SDS-PAGE and quantification showing the expression of pCNA and actin in terminal ileal mucosal scrapings from TLR4-wild-type mice injected with either saline (lanes **i–ii**) or LPS (lanes **iii–iv**) for 3h, * $p < 0.05$. Representative of 3 separate experiments with at least 5 mice per group. **D:** Quantitative RT-PCR showing pCNA expression in terminal ileal mucosal scrapings from TLR4-wild-type and TLR4-mutant mice injected with saline (black

bars) or LPS (white bars) for 3h, * $p < 0.005$ vs. saline in C3H/HeOJ by ANOVA. Representative of 3 separate experiments with at least 5 mice per group. **E:** RT-PCR expression of pCNA in the terminal ileal mucosa of newborn (white bars) and adult mice (checkered bars), and the colon of newborn mice (grey bars) after injection with LPS. * $p < 0.005$ by ANOVA. Representative of 3 separate experiments with at least 5 mice per group.

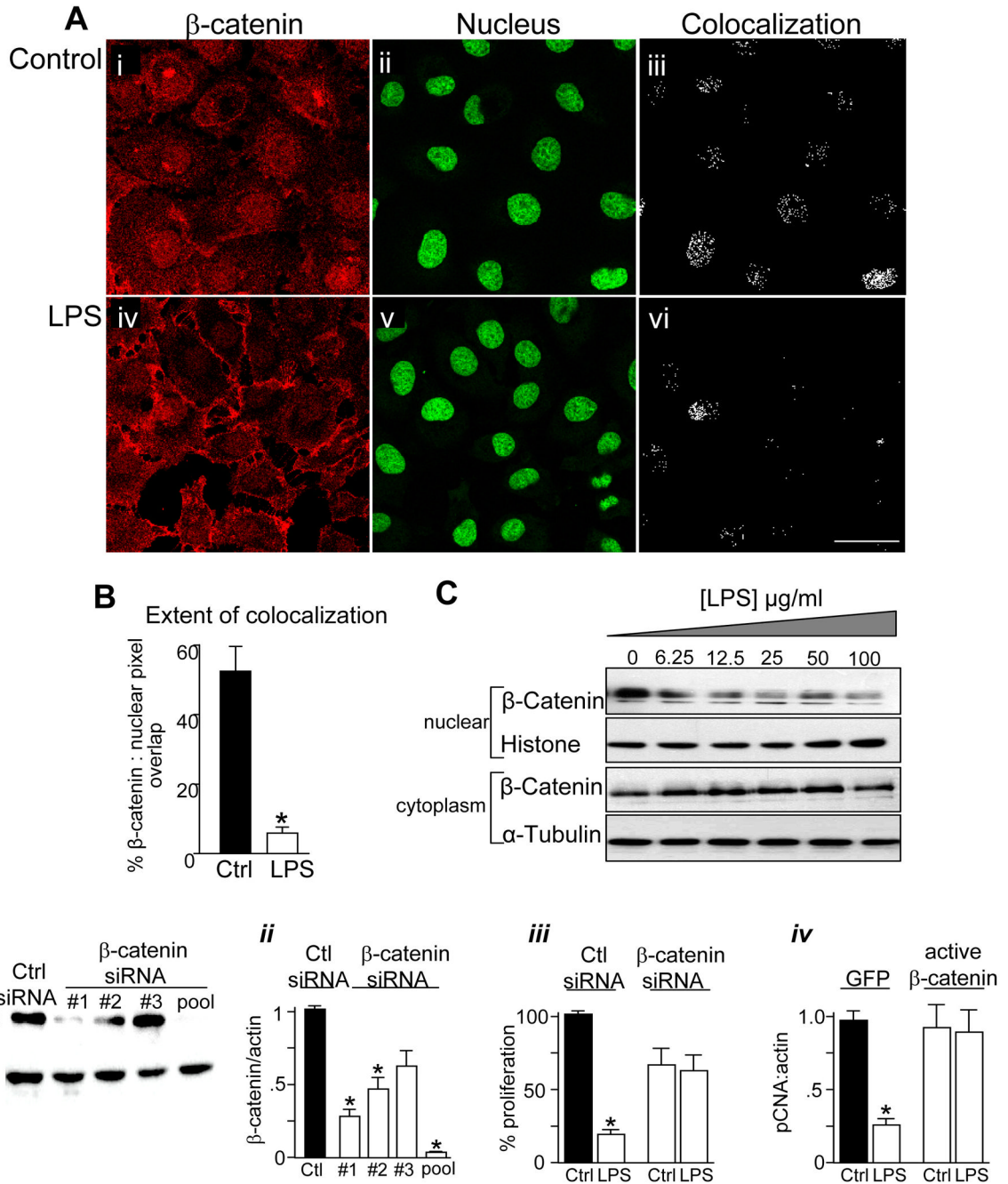


Figure 3. TLR4 activation inhibits enterocyte proliferation via inhibition of β -catenin signaling

A: Confocal images of IEC-6 cells showing β -catenin (red, **i**, **iv**) and the nuclear marker Draq-5 (green, **ii**, **v**). Size bar=10 μ m. **B:** Extent of colocalization (in **iii** and **vi**) was calculated using ImageJ, * $p < 0.05$, representative of 5 separate experiments. **C:** SDS-PAGE of β -catenin in nuclear and cytoplasmic fractions obtained from IEC-6 cells treated with LPS as indicated, along with the nuclear marker histone and the cytoplasmic marker α -tubulin. Representative of 3 separate experiments. **D:** SDS-PAGE (**i**) and quantification of β -catenin:actin expression (**ii**) in IEC-6 cells transfected with siRNA to no known target ("Ctrl") or to β -catenin. Three siRNA's (#1, #2, #3) were used either individually or in combination ("pooled"), as indicated. **iii:** Proliferation in IEC-6 cells treated with the indicated siRNA (XTT assay). **iv:** RT-PCR

showing fold change in pCNA:actin in IEC-6 cells transfected with either GFP or constitutively active β -catenin and treated with LPS. * $p < 0.05$ vs. control, representative of 3 separate experiments.

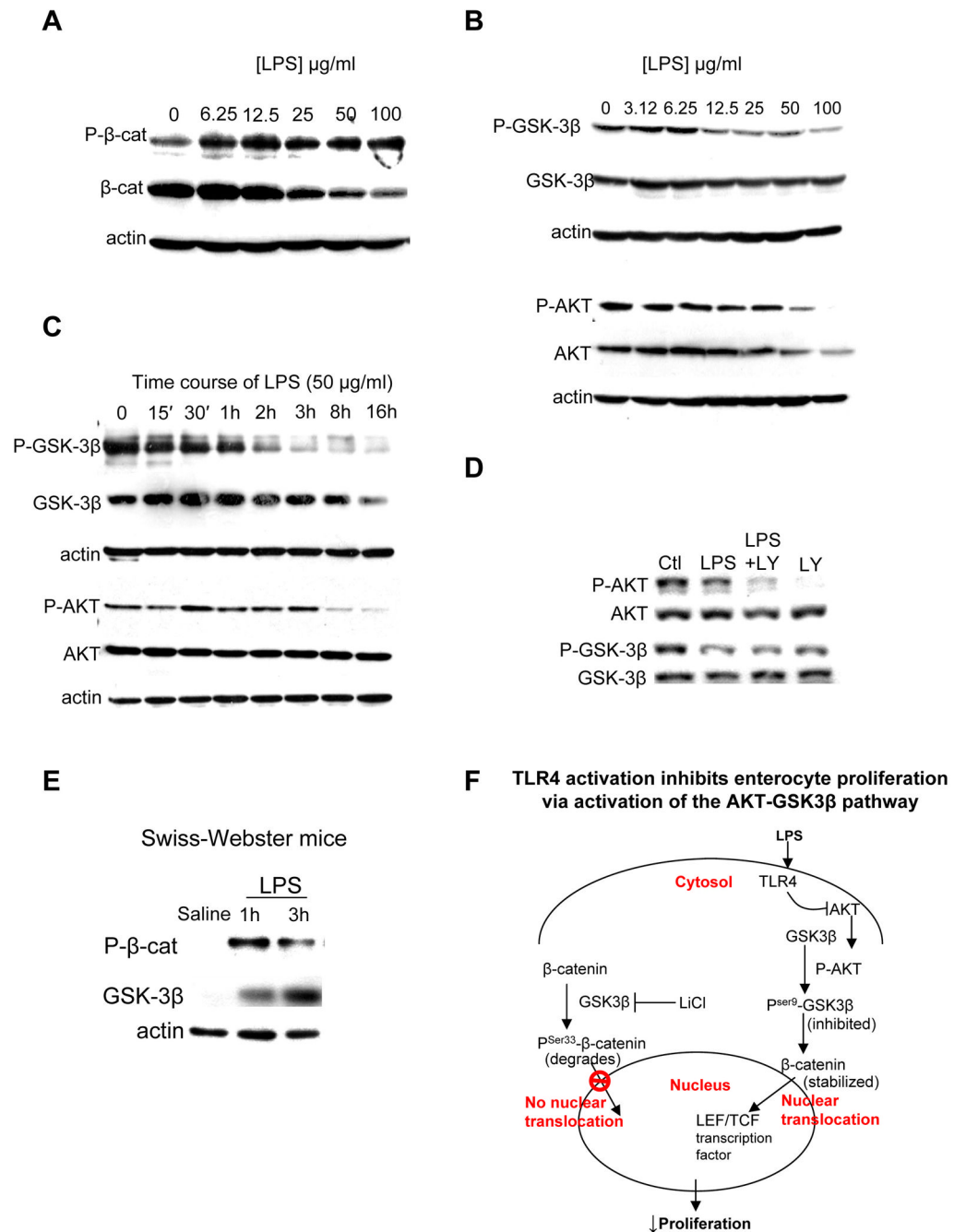


Figure 4. LPS activates the AKT-GSK3 β signaling pathway in enterocytes

A–E: SDS-PAGE showing expression of the indicated protein in IEC-6 cells treated with LPS at the concentrations and durations indicated (**A–D**) or mucosal scrapings from the terminal ileum of mice injected with saline or LPS for the durations indicated (**E**). All blots are representative of 3 separate experiments; over 5 mice per group in **E**. **F:** Schematic summarizing panels **A–D** showing mechanism by which TLR4 inhibits enterocyte proliferation.

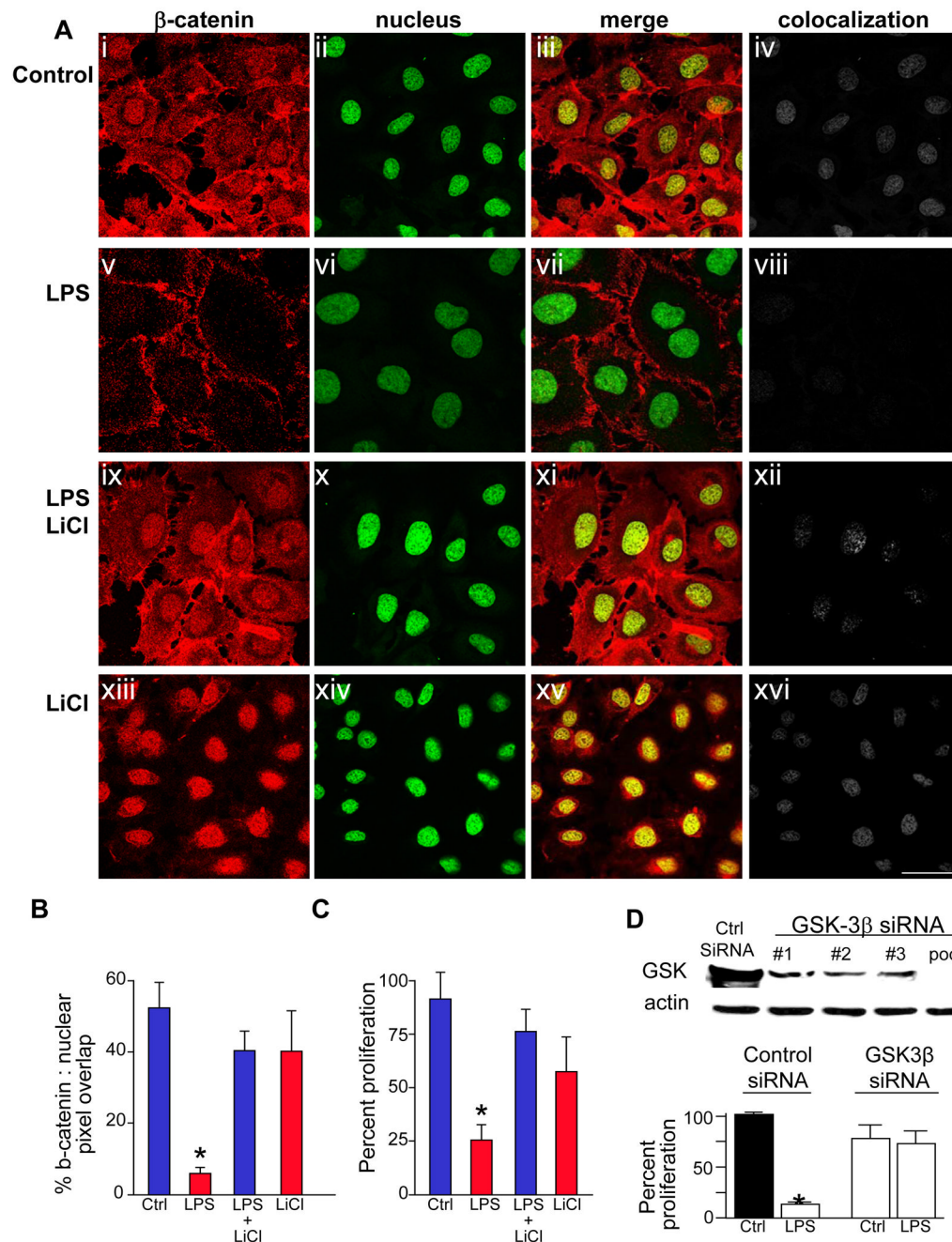


Figure 5. TLR4 activation inhibits enterocyte proliferation via GSK3 β

A: Confocal images showing immunolocalization of β -catenin (red, **i, v, ix, xiii**), the nuclear marker draq-5 (**ii, vi, x, xiv**), merged images (**iii, vii, xi, xv**) and colocalization of nuclear and β -catenin (**iv, viii, xii, xvi**) using ImageJ in IEC-6 cells treated with media (control), or the indicated treatment (LPS and/or the GSK3 β inhibitor LiCl). Size bar=10 μ m. **B:** Quantification of colocalization between the nucleus and β -catenin using ImageJ. * p <0.05 vs. control cells, ANOVA. **C:** Proliferation of IEC-6 cells treated as indicated (XTT assay) * p <0.05 vs. control cells, ANOVA. **D: upper:** SDS-PAGE showing GSK3 β in IEC-6 cells transfected with indicated siRNA's to GSK3 β , either alone (#1,#2,#3) or in combination ("pool"). **lower:**

proliferation of IEC-6 cells (XTT) after treatment with indicated siRNA and LPS or media (“ctrl”)* $p < 0.01$ vs. control cells, ANOVA. Representative of 3 separate experiments.

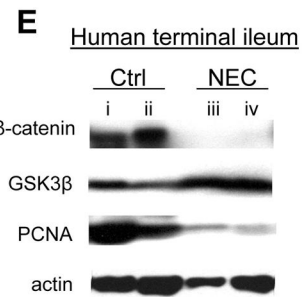
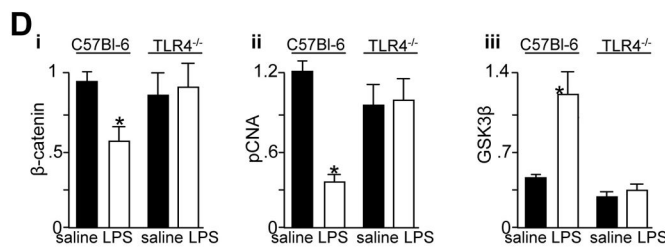
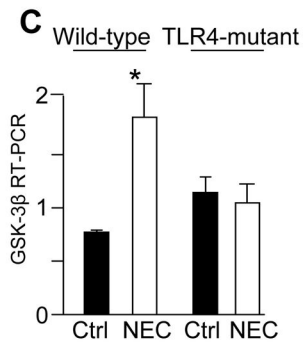
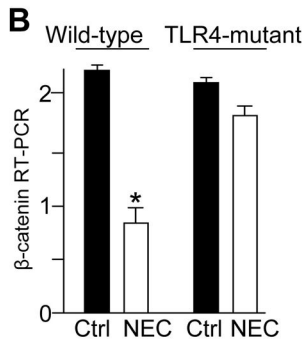
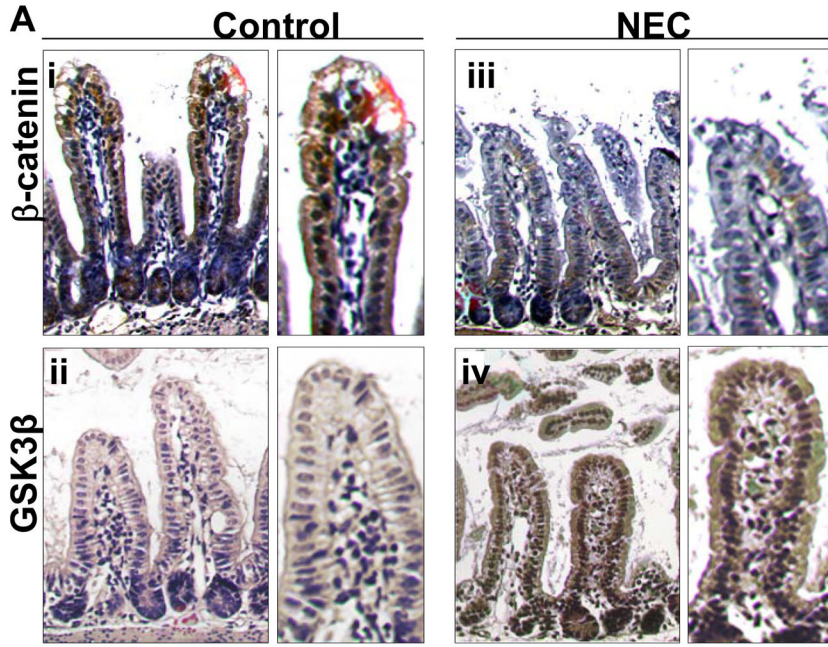


Figure 6. Necrotizing enterocolitis is associated with decreased β -catenin and increased GSK3 β expression in enterocytes in mice and humans

A: Immunostaining of β -catenin (brown staining, **i, iii**) and GSK3 β (brown staining, **ii, iv**) in either breast fed or mice with NEC. Representative of 4 separate experiments with at least 5 mice per group. **B–C:** Quantitative RT-PCR showing β -catenin (**B**) and GSK3 β (**C**) in wild-type and TLR4-mutant mice with (white bars) and without (black bars) NEC. Representative of 3 separate experiments with at least 5 mice per group. **D:** RT-PCR showing the intestinal mucosal expression of pCNA, GSK3 β and β -catenin in wild-type (C57Bl-6) and TLR4^{-/-} mice after injection of saline (black bars) or LPS (white bars). *p<0.05, ANOVA. Representative of 3 separate experiments with at least 5 mice per group. **E:** SDS-PAGE showing β -catenin in

mucosal scrapings obtained from two infants without (lanes i, ii) and two infants with (lanes iii, iv) NEC. Blots were stripped and re-probed for GSK3 β , pCNA and actin.

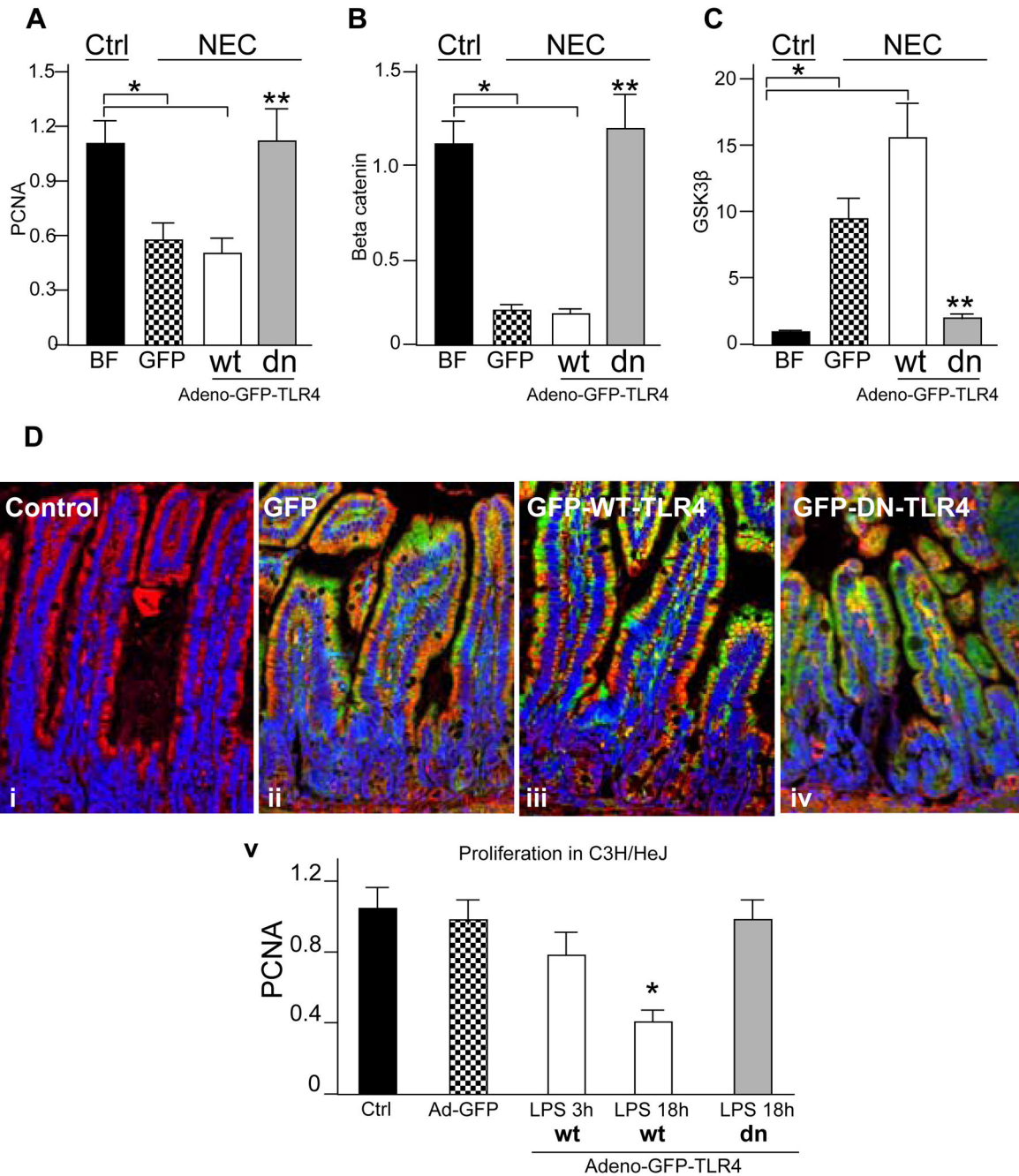


Figure 7. Inhibition of enterocyte TLR4 signaling in mice with NEC restores enterocyte proliferation and reverses the changes in β -catenin and GSK3 β expression

A–C: Newborn mice were either breast fed (black bars) or were induced to develop NEC and administered adenoviruses expressing GFP (checkered bars), GFP-wild-type TLR4 (white bars), or GFP—dominant-negative TLR4 by enteral gavage (grey bars). The expression by quantitative RT-PCR of pCNA (**A**), β -catenin (**B**) and GSK3 β (**C**) in mucosal scrapings in each group is shown. * $p < 0.005$ vs. breast fed, ** $p < 0.001$ vs. both NEC mice administered adeno-GFP and adeno-GFP-wild-type-TLR4, ANOVA. Representative of 5 separate experiments with over 5 mice per group **D: i–v:** Confocal micrographs showing GFP (green), actin (red) and nuclei (blue) in untreated (“control”) mice, or mice treated by gavage with the indicated

adenovirus. **v:** RT-PCR of pCNA in TLR4 mutant mice that were administered GFP (checkered bars), GFP-wild-type-TLR4 (white bars), GFP-dominant-negative-TLR4 (grey bars) or control (black bars) and LPS as indicated. Representative of 3 separate experiments with over 10 mice per group.