Antenatal ureaplasma infection impairs development of the fetal ovine gut in an IL-1-dependent manner

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Ureaplasma infection of the amniotic cavity is associated with adverse postnatal intestinal outcomes. We tested whether interleukin-1 (IL-1) signaling underlies intestinal pathology following ureaplasma exposure in fetal sheep. Pregnant ewes received intra-amniotic injections of ureaplasma or culture media for controls at 3, 7, and 14 d before preterm delivery at 124 d gestation (term 150 d). Intra-amniotic injections of recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra) or saline for controls were given 3 h before and every 2 d after Ureaplasma injection. Ureaplasma exposure caused fetal gut inflammation within 7 d with damaged villus epithelium and gut barrier loss. Proliferation, differentiation, and maturation of enterocytes were significantly reduced after 7 d of ureaplasma exposure, leading to severe villus atrophy at 14 d. Inflammation, impaired development and villus atrophy of the fetal gut was largely prevented by intra-uterine rhIL-1ra treatment. These data form the basis for a clinical understanding of the role of ureaplasma in postnatal intestinal pathologies.

INTRODUCTION

Preterm birth is the primary cause of infant mortality and morbidity¹ and the occurrence of preterm birth has increased in the last decade.² The majority of preterm births is associated with chorioamnionitis, an infection of the fetal membranes, as documented by histological findings or culture.¹ Ascending microbial invasion of the uterine cavity is considered to be the most frequent route of infection.³ Consistently, *Ureaplasma parvum* (UP), a commensal of the urogenital tract of humans is the most common microorganism associated with chorioamnionitis.^{4,5} As the amniotic fluid along with organisms and inflammatory products are swallowed by the fetus, the gastrointestinal tract is a possible route of fetal exposure that may result in fetal gut inflammation. Recently, an association was reported between ureaplasma colonization of preterm infants and an increased incidence of necrotizing enterocolitis, a serious gastrointestinal disorder frequently affecting preterm

neonates.⁶ However, the mechanisms by which ureaplasma colonization can result in inflammation and injury of the preterm gut remains essentially unstudied.

Using a translational chorioamnionitis model in fetal sheep, we recently reported that intra-amniotic lipopolysaccharide resulted in fetal gut inflammation and subsequent altered maturation of the gut barrier and innate immune defences.⁷ Moreover, the harmful inflammatory response of the fetal gut to lipopolysaccharide-induced chorioamnionitis could be largely reproduced by intra-amniotic interleukin-1 (IL-1) injection.⁸ We now hypothesize that ureaplasma infection *in utero* causes immune activation and concomitant disturbances of fetal gut development by IL-1-dependent mechanisms. We therefore administered intra-amniotic UP *serovar* 3 for 3, 7, or 14 d before delivery in the presence or absence of the recombinant human IL-1 receptor antagonist (rhIL-1ra) to inhibit signaling by IL-1 α and IL-1 β ^{9,10} (see Methods for

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experimental design). The effects of ureaplasma with or without rhIL-1ra on fetal gut inflammation, maturation, differentiation, and the intestinal barrier disturbance were assessed.

RESULTS

Inflammation

The infection of the amniotic cavity by ureaplasma resulted in inflammation of the fetal gut. In control fetuses, a few myeloperoxidase-positive (MPO+) cells were detected in the preterm gut and the number of infiltrating MPO+ cells did not change after 3-d exposure to ureaplasma (Figure 1a). However, the number of MPO+ cells increased significantly in fetuses exposed to ureaplasma for 7 and 14 d when compared with controls of the same gestational age (GA). Intra-amniotic injection of rhIL-1ra before ureaplasma tended to decrease the MPO+ cells in the fetal gut both at 7 and 14 d after ureaplasma exposure, but changes were not statistically significant (Figure 1a). MPO counts in control animals that received culture medium remained unaltered by rhIL-1ra pre-treatment.

Ureaplasma exposure induced the influx of CD3+ and CD4+ T cells, which largely paralleled the influx of MPO+ cells. CD3+ and CD4+ T cells were increased in fetuses

exposed to ureaplasma for 7 and 14 d when compared with controls. This increased influx at 7 and 14 d after ureaplasma treatment was blocked by rhIL-1ra (Figure 1b,c). We analyzed the expression of Foxp3, a prototypic marker of regulatory T cells which are essential for immune homeostasis in the intestinal tract.^{11,12} When compared with control animals, FoxP3-positive cells decreased 3, 7 and 14 d after UP exposure (Figure 1d). The depletion of FoxP3+ cells was not changed by rhIL-1ra exposure.

Toll-like receptor mRNA levels

The innate immune receptors responsible for sensing and signaling of *Ureaplasma spp* include Toll-like receptor 2 (TLR2)–TLR6¹³ and TLR2–TLR4.¹⁴ The mRNA levels of TLR2 and TLR6 tend to increase in the fetal gut at 3 d after ureaplasma exposure, but changes were not statistically significant (Figure 2). TLR1, 2, 4, 6, and 9 mRNA levels were all increased at 7 d post-ureaplasma exposure and these increased mRNA levels were inhibited by intra-amniotic rhIL-1ra injection (Figure 2).

Gut damage and morphological changes

We determined whether the inflammatory response of the fetal gut after intra-amniotic ureaplasma exposure for 7 and 14 d

Figure 1 Distribution of inflammatory cells. For each experimental group, (a) myeloperoxidase (MPO)-, (b) CD3-, (c) CD4- and (d) FoxP3-positive cells were counted per high-power field and the average value of the sum of three representative areas is given. UP, *Ureaplasma parvum*.

Figure 2 Quantification using real-time PCR assays using sheep-specific primers and Taqman probes. The values for each Toll-like receptor (TLR) were normalized to 18S rRNA. The mean mRNA signal in controls was given the value of 1 and levels at each time point were expressed relative to controls. All TLRs that were assessed increased after 7 d of *Ureaplasma parvum* (UP) exposure. This increased expression was inhibited by intra-amniotic recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra) injection. The control group and the control rhIL-1ra group each consisted of $n=7$ animals, the 3-d ureaplasma group and the 3-d ureaplasma + rhIL-1ra group each of $n=6$, and the 7-d ureaplasma group and the 7-d ureaplasma + rhIL-1ra group of $n=5$.

resulted in subsequent intestinal damage and morphological changes. Hematoxylin and eosin staining revealed that ureaplasma exposure for 7 and 14 d caused substantial injury to the enterocytes lining the tips of the villi (**Figure 3**). Damage to these vacuolated enterocytes, which are characteristic for the immature gut,^{15,16} was most prevalent in the 7-d-treated animals (**Figure 3b**). This sloughing of cells from the villi resulted in accumulation of luminal debris in the proximity of the injured villi (**Figure 3b**). There was variation of both the intensity and location of the damage to the villi among the ureaplasma-exposed lambs. In a single animal, there were patches where only a few cells were lost from the tips of the villi, whereas in adjacent regions, almost every villus was partially denuded of epithelium. Damage to the villus epithelium and accumulation of luminal debris as seen after intra-amniotic

ureaplasma injection was not prevented by blockage of IL-1 (**Figure 3c**). Besides loss of the villus epithelium, significant villus shortening was observed in the 14-d ureaplasma group (**Figure 3d,f**). The reduced villus length in ureaplasma-exposed lambs was largely prevented by rhIL-1ra and comparable with media-treated control fetuses (**Figure 3e,f**).

The macroscopic damage to the villus epithelium was further substantiated by a disturbed distribution of the tight junction protein ZO-1 (*Zonula occludens* protein 1), a critical component of the paracellular barrier. As reported previously, ZO-1 staining was fragmented in premature control animals.^{7,8} Fetuses exposed to ureaplasma for 7 or 14 d had a more fragmented ZO-1 distribution when compared with control animals, with the most severe disturbance in the 7-d group (**Figure 4**). The disturbed distribution of ZO-1 in ureaplasma-exposed lambs

Figure 3 Cellular damage was determined by morphological analysis of sections that were stained for hematoxylin and eosin. (a) No damage was seen in control samples. (b) In 7-d *Ureaplasma parvum* (UP)-treated fetuses, severe damage of the villus tips (arrow head) and accumulation of luminal debris (arrow) was observed, (c) which was not prevented by blockage of IL-1 signaling. (d) Exposure to UP for 14 d resulted in damage of the villi. (d + f) The villus length decreased after 14 d, but not after 7 d of UP exposure (b + f). (e + f) Compared with time-matched UP-treated fetuses, intra-amniotic recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra) injection resulted in an increased villus length at 14 d. The control group and the control rhIL-1ra group each consisted of $n = 7$ animals, the 3-d ureaplasma group and the 3-d ureaplasma + rhIL-1ra group each of $n = 6$, the 7-d ureaplasma group and the 7-d ureaplasma + rhIL-1ra group each of $n = 5$, and the 14-d ureaplasma group and the 14-d ureaplasma + rhIL-1ra group each of $n = 5$.

Figure 4 Localization of the tight junctional protein ZO-1. (a) ZO-1 staining was fragmented in premature control animals. Fetuses exposed to *Ureaplasma parvum* (UP) for (b) 7 or (d) 14 d had more fragmented ZO-1 when compared with controls. The distribution of ZO-1 in (c) 7-d and (e) 14-d UP-treated fetuses was comparable with controls upon recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra) treatment. Original magnification $\times 400$. For inset, original magnification $\times 1,000$ was used.

was completely prevented by rhIL-1ra and was comparable with media-treated control fetuses (**Figure 4**).

Proliferation, differentiation, and maturation of the fetal gut

In order to understand the mechanism behind the shortening of the villi, we analyzed the frequency and distribution of the four epithelial cell types that populate the fetal intestinal crypts and villi, i.e., mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, paneth cells, and enterocytes following ureaplasma treatment. Fetal ureaplasma exposure did not change the numbers of goblet cells (**Supplementary Figure S1** online) at any time point as assessed by periodic acid-Schiff (PAS) staining. However, in control fetuses, the frequency of PAS-negative goblet cells was substantially higher compared with ureaplasma-treated fetuses, indicating that the mucin composition shifts from neutral (pink cells) to acidic (blue cells) following ureaplasma exposure (**Supplementary Figure S1** online).

Paneth cells were not detectable after staining for PAS/Alcian blue or lysozyme. After 7 and 14 d of ureaplasma exposure, endocrine cells were primarily located in the crypts (**Figure 5b,d**), which was in contrast to the normal distribution along the crypt–villus axis in media-treated fetuses (**Figure 5a**). In addition, a moderate decrease of endocrine cells was observed in lambs exposed to ureaplasma for 14 d (**Figure 5d,e**). RhIL-1ra did not prevent the change of PAS-positive into PAS-negative cells (**Supplementary Figure S1** online). However, the altered distribution of endocrine cells was restored by rhIL-1ra (**Figure 5c,e**). In contrast to goblet cells and endocrine cells, a substantial

decrease of enterocytes was observed in 14-d ureaplasma-treated animals when compared with control animals, and this effect was counterbalanced by rhIL-1ra (**Figure 6**). In summary, these findings implicate that a reduced number of enterocytes is responsible for the villus shortening as seen in 14-d post-ureaplasma exposure.

To gain insight whether the reduced enterocyte number was caused by impaired proliferation, ileal tissue was stained for the proliferation marker KI67 and the mitotic marker Phospho-Histone H3. Fetuses exposed to ureaplasma for 7 or 14 d had fewer proliferating crypt cells (**Figure 7a–e**) and mitotic cells (**Figure 7f**) when compared with controls. This decrease of proliferating and mitotic cells could be blocked by rhIL-1ra treatment, although this effect on the number of mitotic cells in the 7-d ureaplasma group did not reach statistical significance.

The mislocalization and small decrease of endocrine cells and the reduced number of enterocytes prompted us to investigate the distribution of Kruppel-like factor 5 (KLF5), a transcription factor known to be involved in maintenance of epithelial proliferation, differentiation, and cell positioning along the crypt radial axis.¹⁷ Consistent with previous reports,¹⁸ constitutive KLF5 expression was enriched in intestinal epithelial cells located in the lower-to-middle crypt region (**Figure 8a**). In 7-d ureaplasma-treated animals, reduced numbers of KLF5+ cells were detected when compared with media-treated controls, and these KLF5-expressing cells were restricted to the bottom of the crypts (**Figure 8b**). In lambs exposed to ureaplasma for 14 d, the majority of KLF5+ enterocytes were identified at the bottom of the crypts, but in these animals, KLF5-expressing cells were also found in the

Figure 5 Distribution of endocrine cells. (a) Endocrine cells were located using anti-Chromogranin A. In control animals, endocrine cells are normally distributed along the crypt–villus axis (a). After (b) 7-d and (d) 14-d *Ureaplasma parvum* (UP) exposure, endocrine cells were primarily located in the crypts. (c, e) The deviated distribution of endocrine cells in 7- and 14-d UP-exposed fetuses was restored upon intra-amniotic recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra) injection. The number of endocrine cells was slightly reduced in 14-d UP- (d) and 14-d UP + rhIL-1ra-treated (e) lambs. Original magnification $\times 100$.

upper part of the villus (**Figure 8d**). The altered KLF5 distribution following ureaplasma exposure could be blocked by rhIL-1ra treatment (**Figure 8c,e**).

Figure 6 The absolute number of enterocytes along the crypt-villus axis decreased after 14 d but not after 7 d of *Ureaplasma parvum* (UP) exposure. Compared with time-matched UP-treated fetuses, intra-amniotic recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra) injection resulted in increased enterocyte numbers at 14 d. Enterocytes were counted in HE-stained sections per high-power field and the average value of the sum of three representative areas is given. The control group and the control rhIL-1ra group each consisted of $n=7$ animals, the 3-d ureaplasma group and the 3-d ureaplasma + rhIL-1ra group each of $n=6$, the 7-d ureaplasma group and the 7-d ureaplasma + rhIL-1ra group each of $n=5$, and the 14-d ureaplasma group and the 14-d ureaplasma + rhIL-1ra group each of $n=5$.

Consistent with the reduced number of KLF5-expressing enterocytes in the 7-d ureaplasma-treated animals, intestinal fatty-acid-binding protein (I-FABP) expression was reduced or absent in the villus epithelium in 7-d ureaplasma-treated fetuses and this reduced expression was counter-balanced in rhIL-1ra-treated fetuses (**Figure 9**). As the loss of I-FABP expression in the gut following ureaplasma infection did not result in elevated fetal plasma levels for I-FABP (data not shown), these data suggest that diminished differentiation, rather than cell death, is the cause of this phenotypic change of enterocytes. In line with this assumption, ureaplasma exposure did not result in a higher number of apoptotic cells as assessed by caspase-3 staining (not shown).

As the animals are infected not only for different lengths of time but also infected at different stages of development, we tested the proliferation, differentiation, and maturation of the fetal ovine gut from 110–124 d GA in the absence of ureaplasma and or rhIL-1ra. As gut development changed little over the period of 110–124 d of gestation (data not shown), the stage of development will have little influence on the response to ureaplasma or rhIL-1ra.

DISCUSSION

The most important determinant of morbidity in prematurely born neonates is the immaturity of organs.¹⁹ Chorioamnionitis, which exposes the pulmonary and gastrointestinal tracts to microorganisms, is one of the major causes of preterm birth.^{20,21} Antenatal exposure of the lung to microorganisms changes early neonatal lung function and may alter long-term lung function and growth.²² In addition, increasing evidence

Figure 7 Distribution of proliferating cells (Ki-67+) and cell counts of mitotic cells (Phospho-Histone H3+). Compared with (a) controls, fetuses exposed to *Ureaplasma parvum* (UP) for (b) 7 d or (d) 14 d contain less proliferating intestinal epithelial cells. These effects after (c) 7 d or (e) 14 d of UP exposure could be blocked by recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra) treatment. Original magnification $\times 200$. (f) For each experimental group, mitotic cells were counted per high-power field and the average value of the sum of three representative areas is given.

Figure 8 Distribution of KLF5 + cells. Compared with (a) controls, fetuses exposed to *Ureaplasma parvum* (UP) for (b) 7 d and (d) 14 d have reduced numbers of KLF5 + cells in the crypt–villus axis (red bars). Reduced KLF5 + cell numbers were not observed in atrophic villi in 14-d UP-treated fetuses (d) (yellow bar). The reduction of KLF5 + cells in (c) 7-d and (e) 14-d UP-treated animals could be blocked by recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra) treatment. Original magnification $\times 200$.

Figure 9 Localization of I-FABP. (a) Intestinal fatty-acid-binding protein (I-FABP) is located along the villus in control animals. (b) Expression in 7-d *Ureaplasma parvum* (UP)-treated fetuses is confined to intestinal crypts. (c) This reduced expression was counterbalanced in recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra)-treated fetuses. (d) In 14-d UP-treated animals, I-FABP expression is expressed by all cells lining the villi. (e) Villus atrophy and the distribution of I-FABP-expressing enterocytes in 14-d UP-exposed fetuses was restored upon intra-amniotic rhIL-1ra injection. Original magnification $\times 200$.

shows that chorioamnionitis interferes with intestinal development and is associated with adverse intestinal outcomes.^{6–8,23,24} This study reports the novel finding that *in utero* exposure to UP,

the most common microorganism associated with chorioamnionitis, results in fetal gut inflammation, cellular damage to the villus epithelium, accumulation of luminal debris, and disturbed

distribution of the intracellular tight junctional ZO-1. Furthermore, ureaplasma colonization of the fetus led to substantial architectural changes of the crypt–villus axis. In particular, proliferation, differentiation, and maturation of enterocytes were significantly perturbed as illustrated by decreased proliferation and a strong reduction of KLF5- and I-FABP-expressing enterocytes. Interestingly, these early changes resulted in severe villus atrophy after ureaplasma exposure for 14 d. Importantly, our results strongly link IL-1 activation with inflammation and underdevelopment of the intestine by demonstrating strong protective effects of prenatal rhIL-1ra administration in the course of ureaplasma infection. In ureaplasma-exposed fetuses, rhIL-1ra treatment resulted in less intestinal inflammation, improvement of the barrier integrity, restored proliferation, differentiation and maturation of enterocytes and prevention of villus atrophy.

An increased pro-inflammatory status, disturbed barrier integrity and intestinal immaturity are considered to underlie necrotizing enterocolitis pathology.^{25–28} Therefore, our findings provide a mechanistic link for the clinical observation that ureaplasma colonization of the fetus is associated with an increased risk of necrotizing enterocolitis development.⁶ Additionally, immaturity of intestinal epithelial cells and the decreased surface area of villi could result in maldigestion and poor mucosal protection against colonizing bacteria in the postnatal period.^{29,30}

Different mechanisms may underlie the protective effects of rhIL-1ra in ureaplasma-treated animals. First, our data suggest that rhIL-1ra-dependent recovery of the disturbed crypt–villus architecture, enterocyte immaturity and villus atrophy in ureaplasma-exposed fetuses are the result of the recuperation of epithelial proliferation. Second, rhIL-1ra treatment prevented T-effector cell expansion and the associated detrimental inflammatory response in ureaplasma-exposed fetuses, potentially by improvement of the apparent CD4 + /FoxP3 + imbalance. Consistent with this theory, a regulatory T cell-to-T-effector cell imbalance has been recently associated with an increased incidence of necrotizing enterocolitis, confirming that such a pro-inflammatory environment could lead to severe inflammation and damage of the preterm ileum.³¹

Third, rhIL-1ra completely prevented the ureaplasma-driven increase of TLRs, key receptors of the innate immune system which respond to a large group of pathogen-associated molecular patterns, including lipoproteins from the *Ureaplasma* spp.^{13,14} These data suggest that increased TLR signaling is causally linked with the detrimental inflammatory response in the intestine after ureaplasma exposure. However, the proteins that mediate signaling from these receptors, such as MyD88 and IL-1 receptor-associated kinase-4, have also been shown to have important roles in the response to microorganisms and warrant further investigation.³²

Interestingly, the IL-1 β protein was detected by an ovine IL-1 β -specific enzyme-linked immunosorbent assay (ELISA) in ileal tissue, whereas no IL-1 β was detected in any of the amniotic fluid samples (data not shown). This result showed that the IL-1-dependent inflammatory and developmental

changes in the fetal gut following ureaplasma exposure were a direct response of the fetus rather than an indirect result of oral intake of IL-1-containing amniotic fluid.

The increased influx of MPO + -expressing cells, damage to the villus epithelium, and accumulation of luminal debris following intra-amniotic ureaplasma injection was not prevented by blockage of IL-1 signaling. This indicates that IL-1-independent mechanisms may also be responsible for some of the detrimental effects of ureaplasma colonization of the amniotic fluid.

Although the net result of an inflammatory response is the recruitment of inflammatory cells such as seen after 7 d after ureaplasma treatment, the current experimental set up does not allow us to study the inflammatory response in more detail between 3 and 7 d after ureaplasma. Future studies have to take this into account in addition to the complex effects that the inhibition of IL-1 receptor signaling may have on the multiple inflammatory events in the fetal ileum.

Although our data suggest that rhIL-1ra has potential therapeutic use for ureaplasma-induced fetal gut inflammation, it is currently unknown whether rhIL-1ra can be safely used in the human fetus. A limitation of the current study is that there is no insight into the effects of long-term ureaplasma exposure on fetal gut inflammation and subsequent development. Future research is warranted to investigate whether fetal gut damage and interference with maturation further progresses or resolves after longer ureaplasma exposure of the fetus. In addition, future studies should focus on the postnatal intestinal changes after intrauterine ureaplasma exposure and the possible development of intestinal pathologies after birth.

The important conclusion from the present study is that intrauterine ureaplasma exposure results in fetal gut inflammation, intestinal injury and barrier loss, and impaired maturation. Moreover, these ureaplasma-induced effects on fetal gut inflammation and development are largely IL-1 dependent. Our data suggest that administration of rhIL-1ra, which is used to treat immune disorders such as arthritis, could be a potential clinical tool to prevent the adverse intestinal outcomes upon birth after chorioamnionitis.

METHODS

Experimental protocol. The animal studies were approved by the Animal Ethics Committee of The University of Western Australia, Perth, WA, Australia and the Children's Hospital Medical Center, Cincinnati, OH. Ureaplasmas were grown and prepared for ultrasound-guided injections as reported previously.³³ Time-mated Merino ewes with singleton fetuses were randomly assigned to groups of five to seven animals to receive UP *serovar* 3 (2×10^7 colony-forming units (CFU)) or media control at 110, 117, or 121 d GA (term = 150 d GA; **Figure 10**). Colonization of the amniotic fluid with UP 3 was confirmed in all ureaplasma-exposed lambs with $> 10^7$ cfu ml⁻¹ at delivery. Colonization was not detected in media controls. Using the same ovine model, we previously demonstrated that injection of UP in the amniotic fluid resulted in chronic colonization of the amniotic fluid, the fetal lung and gut.^{34,35} IL-1 signaling was inhibited with rhIL-1ra (anakinra (Kineret); Amgen, Thousand Oaks, CA). Intra-amniotic injections of 100 mg rhIL-1ra resuspended in 2 ml saline or the equivalent volume for control animals were given 3 h before the

Figure 10 Ewes bearing singleton fetuses were randomly assigned to groups of five to seven animals to receive *Ureaplasma parvum* serovar 3 (2×10^7 colony-forming units) or the suspending medium alone at 110, 117, or 121 d gestational age (GA). Interleukin (IL)-1 signaling was inhibited with recombinant human interleukin IL-1 receptor antagonist (rhIL-1ra). Intra-amniotic injections of 100 mg rhIL-1ra or saline for control animals were given 3 h before the intra-amniotic ureaplasma or media injections. Animals were then treated every 2 d with additional intra-amniotic injections of 100 mg rhIL-1ra or saline.

intra-amniotic ureaplasma or media injections. Animals were then treated every 2 d with additional intra-amniotic injections of 100 mg rhIL-1ra or saline (**Figure 10**). This dose and route of administration of rhIL-1ra completely inhibited intra-amniotic IL-1 α -induced lung and systemic inflammation (reported in the online section of Kallapur *et al.*³⁶). Intra-amniotic rhIL-1ra also blocked most of the inflammation and lung maturation induced by intra-amniotic lipopolysaccharide.³⁶ At 124 d GA, the ewe was sedated (10 mg kg⁻¹ ketamine, Parnell Labs, Alexandria, Australia; 0.02 mg kg⁻¹ medetomidine, Pfizer Animal Health, West Ryde, Australia) before receiving a regional spinal anesthetic block (2% lignocaine: 3 ml, 60 mg) and delivery of the preterm lamb by cesarean section. The lamb was immediately euthanized (100 mg kg⁻¹ sodium pentobarbitone, Valabarb, Pitman-Moore, Australia) and samples of the distal ileum were collected for snap freezing and fixation.

Antibodies. The following antibodies were used: rabbit antibodies against human Phospho-Histone H3 (Santa Cruz Biotechnology, Santa Cruz, CA), MPO, CD3, lysozyme (all from Dakocytomation, Glostrup, Denmark), ZO-1 (Invitrogen, San Francisco, CA), and activated caspase-3 (Cell Signaling Technology, Danvers, MA); rabbit anti-bovine SP-1 Chromogranin A (ImmunoStar, Hudson, WI); monoclonal antibodies against bovine CD4 (VMRD, Pullman, WA), human FoxP3 (eBioscience, San Diego, CA), and human Ki-67 (Dakocytomation). Antibodies against I-FABP were kindly provided by the Department of surgery, Maastricht University Medical Center, the Netherlands headed by Prof. Steven Olde Damink. A guinea pig antibody against human KLF5 was a kind gift from Dr. Jeffrey Whitsett from the Section of Neonatology, Perinatal and Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH. Secondary antibodies, biotin-conjugated rabbit anti-mouse, goat anti-guinea pig or swine anti-rabbit, and Texas red-conjugated goat

anti-rabbit were purchased from Dakocytomation and peroxidase-conjugated goat anti-rabbit from Jackson (West Grove, PA).

Immunohistochemistry. Formalin-fixed gut tissue was embedded in paraffin and 3 μ m sections were used for immunohistochemistry. For detection of CD3, Ki-67, Phospho-Histone H3, lysozyme, Chromogranin A, Caspase-3 and KLF5-expressing cells, slides were boiled in 10 mM Na-citrate (pH 6.0) for 20 min. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol. Slides were blocked with normal goat serum (MPO, FoxP3, Ki-67, Phospho-Histone H3, lysozyme, Chromogranin A, Caspase-3, KLF5) or bovine serum albumin (CD3, CD4) for 30 min at room temperature. Slides were incubated with the primary antibody of interest for 1 h at room temperature (CD3, CD4, and MPO) or overnight at 4 °C (FoxP3, Ki-67, Phospho-Histone H3, lysozyme, Chromogranin A, Caspase-3, and KLF5). After washing using phosphate-buffered saline, sections were incubated with the appropriate secondary-conjugated antibody. CD3 and FoxP3 antibodies were detected with the streptavidin-biotin system (Dakocytomation); antibodies against MPO and CD4 were detected using a peroxidase-conjugated secondary antibody; antibodies against Ki-67, Phospho-Histone H3, lysozyme, Chromogranin A, Caspase-3, and KLF5 were detected using a biotin-conjugated secondary antibody. Positive staining for MPO, CD3, and CD4 was visualized with 3-amino-9-ethylcarbazole (Sigma, St Louis, MO); nuclei were counterstained with haematoxylin. Immunoreactivity for FoxP3, Ki-67, Phospho-Histone H3, lysozyme, Chromogranin A, Caspase-3, and KLF5 was visualized using nickel-DAB; and nuclei were counterstained with nuclear fast red. The number of cells exhibiting immunostaining were counted per single (MPO, CD3, CD4, Phospho-Histone H3, Chromogranin A, $\times 200$) or three high power fields (FoxP3, $\times 100$). The numbers of enterocytes were counted in sections stained for hematoxylin and eosin ($\times 200$). The immunohistochemical analysis was scored by three investigators who were blinded to the experimental conditions.

PAS/Alcian blue stain. De-waxed sections were immersed in Alcian Blue 8GX staining solution (pH 2.5; Richard Allan Scientific, MI) for 5 min. After washing in distilled water, slides were oxidized in 1% periodic acid (Richard Allan Scientific) in water at room temperature for 5 min, washed in distilled water for 5 min, immersed in Schiff's reagent (Richard Allan Scientific) for 5 min, and rinsed in tap water for 10 min. Following the three washing steps in distilled water, slides were counterstained in hematoxylin for 3 min and dipped three times in phosphate-buffered saline. Then, slides were dehydrated and coverslipped.

Immunofluorescence for ZO-1. Immunofluorescence was performed and interpreted as described earlier.⁷ The ZO-1 distribution was recorded at a magnification of $\times 200$ using the Metasystems Image Pro System (black and white charge-couple device camera; Metasystems, Sandhausen, Germany) mounted on a Leica DM-RE fluorescence microscope (Leica, Wetzlar, Germany).

TLR mRNA quantitation. Ileal total RNA was isolated and reverse transcribed into cDNA as previously described.³⁷ cDNA was used as a template with primers and Taqman probes (Applied Biosystems, Carlsbad, CA) specific to sheep sequences. The values for each TLR were normalized to the internal RPS15 rRNA value. Data were expressed as fold increase over the control value. Primer sequences are provided in the **Supplementary Table S1** online.

ELISA. Differences of plasma I-FABP levels were determined by ELISA to measure intestinal mucosal cell damage.³⁸ For this purpose, a high-sensitive ELISA was used that was kindly provided by the Department of surgery, Maastricht University Medical Center, the Netherlands headed by Prof. Steven Olde Damink. A similar protocol was used as previously described.³⁹

Statistical analysis. The number of cells exhibiting immunostaining for the protein of interest were counted per high power field. Normality of all data obtained was evaluated by Kolmogorov–Smirnov test. Overall comparisons among groups were performed by Kruskal–Wallis testing. Then individual between group comparisons were made using post-hoc Dunn’s multiple comparison testing. Data are presented as median and range. Statistical calculations were made using SPSS 15.0 for Windows (SPSS, Chicago, IL) and differences were considered statistically significant at $P < 0.05$.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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