A Mouse Model of Pathological Small Intestinal Epithelial Cell Apoptosis and Shedding Induced by Systemic Administration of Lipopolysaccharide

Running Title: LPS Induced Epithelial Shedding

Jonathan M. Williams,1 Carrie A. Duckworth,1 Alastair J. M. Watson,2 Mark R. Frey,3 Jennifer C. Miguel,3 Michael D. Burkitt,1 Robert Sutton,4 Kevin R. Hughes,2,6 Lindsay J. Hall,2,6 Jorge H Caamaño,5 Barry J. Campbell,1 D. M. Pritchard1*

1 Department of Gastroenterology, Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom

2 Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, United Kingdom

3 Departments of Pediatrics and Biochemistry & Molecular Biology, The Saban Research Institute at Children's Hospital Los Angeles, University of Southern California, United States

4 NIHR Liverpool Pancreas Biomedical Research Unit, 5th Floor UCD Block, Royal Liverpool University Hospital, Daulby Street, Liverpool, United Kingdom

5 School of Immunity and Infection, University of Birmingham, United Kingdom

6 Institute of Food Research, Norwich Research Park, Colney, Norwich, United Kingdom
* Corresponding author

Correspondence details:
Department of Gastroenterology
Institute of Translational Medicine
Nuffield Building
Crown Street
Liverpool
L69 3GE
Email: dmpritch@liverpool.ac.uk
Tel: +44(0)151 794 5772
Fax: +44(0)151 794 6825

Keywords: Lipopolysaccharide, intestinal epithelium, apoptosis, shedding, receptors tumor necrosis factor, NF-kappa B
Summary

The gut barrier, composed of a single layer of intestinal epithelial cells (IECs) held together by tight junctions, prevents the entrance of harmful microorganisms, antigens and toxins from the gut lumen. Small intestinal homeostasis is normally maintained by the rate of shedding of senescent enterocytes from the villus tip exactly matching the rate of generation of new cells in the crypt. However in various localized and systemic inflammatory conditions, intestinal homeostasis may be disturbed as a result of increased IEC shedding. Such pathological IEC shedding may cause transient gaps to develop in the epithelial barrier and result in increased intestinal permeability. Although pathological IEC shedding has been implicated in the pathogenesis of conditions such as inflammatory bowel disease, our understanding of the underlying mechanisms remains limited. We have therefore developed a murine model to study this phenomenon, as IEC shedding in this species is morphologically analogous to humans. IEC shedding was induced by systemic lipopolysaccharide (LPS) administration in wild-type C57BL/6 mice, and mice deficient in TNF-receptor 1 (Tnfr1−/−), Tnfr2−/−, Nuclear Factor kappa B1 (Nfb1−/−) or Nfb2−/−. Apoptosis/cell shedding was quantified using immunohistochemistry for active Caspase-3 and gut lumen to systemic circulation permeability was assessed by measuring plasma fluorescence following fluorescein isothiocyanate-dextran gavage. LPS, at doses ≥0.125mg/kg induced rapid villus IEC apoptosis with peak cell shedding occurring at 1.5h. This coincided with significant villus shortening, fluid exudation into the gut lumen and diarrhea. A significant increase in gut to circulation permeability was observed at 5h. TNFR1 was essential for LPS-induced IEC apoptosis and shedding and the fate of the IEC was also dependent on NFκB, with signaling via NFκB1 favoring cell survival, and via NFκB2 favoring apoptosis. This model will enable
investigation of the importance and regulation of pathological IEC apoptosis and cell shedding in various diseases.
Introduction

The gut barrier comprises a single layer of intestinal epithelial cells (IECs) and the tight junctions between them. It allows absorption of nutrients from the intestinal lumen into the circulation, while preventing the entry of injurious microorganisms, toxins, and antigens. In the small intestine, IECs are generated in the crypt, migrate up the villus, and are shed at the villus tip (Leblond and Stevens, 1948). In mice, which exhibit whole IEC shedding similar to that which occurs in humans (Bullen et al., 2006), approximately 1400 epithelial cells are estimated to be shed in this way from a single villus tip per day. The small intestine therefore has one of the highest cell turnover rates in the body, with an estimated $10^{11}$ and $2 \times 10^8$ cells being shed per day from the small intestine of humans and mice respectively (Potten and Loeffler, 1990). During the process of physiological cell shedding, the highly coordinated process of tight junction rearrangement that is required to allow the detachment and release of IECs from the epithelium maintains the gut barrier (Madara, 1990).

In various inflammatory conditions however, the loss of epithelial cells from the villus exceeds the rate of epithelial generation in the crypt. This process, which we have termed ‘Pathological IEC shedding’ remains poorly understood. Such pathological IEC shedding may represent the earliest intestinal injury in a variety of intestinal diseases and is likely to have important consequences, as it may result in gap formation in the epithelium, permeability defects and villus shortening (villus atrophy). Indeed, increased numbers of shedding epithelial cells with corresponding focal permeability defects and epithelial gaps have been observed in inflammatory bowel disease (IBD), including both Crohn’s disease (CD) and ulcerative colitis (UC) (Kiesslich et al., 2012; Liu et al., 2011). It has also been shown that individuals at high risk of developing IBD exhibit increased gastrointestinal
permeability (Hollander et al., 1986). Similarly, IL-10 deficient mice exhibit increased small intestinal permeability prior to the development of spontaneous colitis. In this animal model, colitis severity can be markedly reduced by administering a specific pharmacologic inhibitor which reduces small intestinal permeability by preventing the opening of tight junctions, and is prevented completely by rearing animals in germ-free conditions. This suggests a critical link between small intestinal permeability, luminal antigens and development of chronic colitis. (Arrieta et al., 2009).

While recombinant tumor necrosis factor (TNF) has been previously shown to induce pathological intestinal villus epithelial cell shedding in mice (Kiesslich et al., 2007), the exogenous administration of this cytokine in isolation does not reflect the complexity of the mammalian inflammatory response that is present in most disease states. In addition, the concentrations of exogenous TNF required to induce IEC shedding are higher than found in-vivo, making such experiments expensive and possibly yielding artefactual results. We have also found TNF to be an inconsistent stimulus of IEC shedding. We therefore sought to find a simple, inexpensive, rapid, reproducible and pathologically relevant stimulus to investigate the process of IEC shedding in detail in an animal model.

Lipopolysaccharide (LPS) is an integral component of Gram negative bacteria and is a potent activator of the innate immune system. It represents a pathogen associated molecular pattern (PAMP) recognized by Toll-like receptor 4 (TLR4)(Beutler et al., 2001), which initiates a systemic inflammatory response, with Nuclear Factor kappa B (NFκB) signaling pathways playing a central role in cell responses downstream of both TLR (Chow et al., 1999) and subsequent cytokine receptor ligation (Jacque et al., 2005). We therefore hypothesized that the mammalian systemic inflammatory response was capable of causing epithelial cell
apoptosis and shedding at the villus tip when triggered by LPS and that this occurred prior to the onset of apoptosis in the crypt.

We have therefore examined in detail the earliest phase of LPS induced murine gut injury. We demonstrate that intraperitoneally administered LPS is a simple, rapid, and consistent stimulus of villus IEC apoptosis and cell shedding in the murine small intestine and that this occurs several hours prior to the onset of crypt apoptosis. This early response coincides with fluid effusion into the small intestinal lumen and diarrhea. We have subsequently characterized the dose response and the kinetics of this highly dynamic phenomenon. Using knockout mouse models, we have found that TNFR1-mediated signaling is essential for these events, with an NFκB2 dominant response favoring apoptosis. These data provide interesting insights into the control of IEC homeostasis in inflammatory disease, as the NFκB2 pathway has not previously been linked to IEC apoptosis and shedding.
Results

Systemic LPS caused clinical signs and gross pathological changes from 1.5 hours, with fluid exudation into the intestinal lumen

To establish the time dependent intestinal effects of LPS, we administered 10mg/kg phenol-extracted (PE-LPS) by i.p. injection to adult female WT mice, and euthanized them after 1, 1.5, 2, 3, 4 and 6h. Diarrhea was observed from 1.5-2h. At necropsy, there was serosal pallor of the small intestine (Figure 1A), which exhibited distension with watery yellow fluid. These observations showed that LPS caused acute fluid exudation into the gut lumen with acute onset diarrhea.

LPS caused small intestinal villus IEC loss and cell shedding from 1.5 hours

We performed histopathological examination of H&E stained sections to characterize intestinal injury. At 1.5h (Figure 1A) there was marked villus shortening, clubbing, and blunting. IECs at the villus tip exhibited variable separation and detachment from neighboring cells, often with a teardrop morphology and an apically positioned nucleus (consistent with cell shedding and apoptosis). Large numbers of shed epithelial cells were present within the lumen. Comparable injury was not observed in the stomach, colon, or other organs investigated (Supplementary Figure 1). These observations suggest that LPS causes rapid and specific small intestinal villus epithelial injury, and that peak shedding correlates with the onset of clinical diarrhea.

LPS caused rapid villus shortening with IEC loss in the duodenum, jejunum, and ileum

As villus shortening is commonly utilized as a measure of small intestinal damage, we measured villus heights after LPS administration. In the duodenum 1.5h after LPS
administration, mean villus height was reduced by 29% (Figure 1B) to 260.5±15.0µm compared to untreated (365.9±6.6µm ($P<0.01$: ANOVA)). Reduction in villus height was still evident through to 6h post-LPS (all $P<0.05$: ANOVA). A similar trend was also observed in both the jejunum and ileum, but differences did not reach statistical significance.

Villus shortening was associated with lower numbers of IECs lining the duodenal villi, with a 21% reduction in mean cell number observed at 1.5h post LPS administration (62.7±3.7 versus 78.5±1.8 cells in untreated mice), reaching significance at 3h, with a 40% decrease observed (47.0±4.7 cells, $P<0.05$: Kruskal-Wallis) (Figure 1C). This correlated with large numbers of shed IECs within the intestinal lumen, suggesting that cell shedding occurs contemporaneously with villus shortening.

**LPS significantly increased gut to circulation permeability by 5h**

In order to measure gut to circulation permeability, mice were administered fluorescein-isothiocyanate-conjugated dextran (FD4) by oral gavage, followed by 10mg/kg LPS. At 5h post-LPS (5hrs FD4), there was a 5-fold increase ($P<0.05$: Kruskal-Wallis) in plasma fluorescence, at 76.3±21.7 fluorescent units (Figure 1D) versus 16.4±2.9 in untreated animals (5hrs FD4), suggesting that gut barrier dysfunction allows large molecules to enter the bloodstream at around 5h post-LPS.

**LPS caused activation of Caspase-3 with concomitant apoptosis and cell shedding of villus IECs and relatively spared the crypts**

To investigate the type of cell death responsible for IEC shedding and loss from the villus, we performed IHC for active Caspase-3. Large numbers of villus IECs exhibited positive immunolabeling as early as 1h, with almost universal immunolabeling of shed cells seen
within the small intestinal lumen (Figure 2A). Villus epithelial cells were quantified by microscopy as “apoptotic” or “shedding” (as defined in Materials and Methods and summarized in Figure 2B) and expressed as a percentage of total villus IECs counted.

Maximal active Caspase-3 labeling of 12.5±1.7% villus IECs was found in the duodenum 1.5h after LPS (Figure 2C), representing a 21 fold increase compared to untreated mice (0.6%±0.2%, P<0.05: Kruskal-Wallis). Comparable IEC apoptosis and cell shedding were also observed at 1.5h in the jejunum and ileum (12.1%±2.4% and 11.2%±1.3% respectively). We therefore concluded that LPS caused dynamic villus IEC apoptosis and shedding and that this occurred relatively uniformly throughout the small intestine. The almost universal positive labeling of epithelial cells undergoing shedding additionally suggests that activation of the terminal pathway of apoptosis occurs prior to shedding in this model, rather than being triggered by detachment as occurs during the process of anoikis. Interestingly, crypt IEC apoptosis as interpreted by active Caspase-3 IHC did not show a comparable magnitude of increase to that observed in villi at 1.5h (Figure 2D), although there was a ~3 fold increase by 6h post LPS (1.2%±0.3% versus 0.4%±0.1% in untreated). Accordingly, crypt counts did not alter significantly throughout the time course studied (Figure 2E), in contrast to villus IEC counts.

**LPS induced apoptosis and cell shedding which increased towards the villus tip**

LPS (10mg/kg) increased the number of apoptotic and/or shedding IECs with similar distribution along the villus-axis in the duodenum, jejunum and ileum (Figure 2C). Apoptosis was markedly increased in the apical 50% of the villus, particularly at 1.5h, with a sharp increase in IEC shedding being observed at the villus tip.
**LPS caused maximal apoptosis and shedding at a threshold dose**

We administered 0.125-20mg/kg PE-LPS to WT mice and euthanized them after 1.5h to test whether LPS-induced IEC apoptosis and cell shedding was dose dependent. LPS at 0.125mg/kg caused a minimal (5%) reduction in villus height at 348.1±17.1µm versus 399.0±35.5µm in vehicle-treated control mice (Figure 3A) but with a 10-fold observed increase in IEC apoptosis and cell shedding at 6.0±1.7% ($P<0.05$: ANOVA) (Figure 3B). LPS doses ≥0.25mg/kg caused ~30% reduction in villus height compared to controls, and IEC apoptosis and cell shedding of ~12%. We concluded that LPS-induced small intestinal injury is initiated by a threshold dose of ~0.125mg/kg. Villus IECs therefore appear extremely sensitive to LPS-induced apoptosis and cell shedding, whereas concomitant villus shortening only occurs at higher dosages of LPS.

**LPS purity did not significantly affect IEC apoptosis and cell shedding**

To assess whether LPS purification/extraction method altered IEC apoptosis and cell shedding, we administered high-purity ion-exchange chromatography extracted LPS (IE-LPS;10mg/kg) to WT mice for 1.5h. This preparation caused similar villus shortening to 10mg/kg PE-LPS (298.0±39.1µm compared to 260.5±36.8µm)(Figure 3C). IEC apoptosis and cell shedding post IE-LPS administration were also significantly increased (10.8±2.8%) as observed for PE-LPS (12.5±1.7%) (Figure 3D).

**LPS induced apoptosis and cell shedding was significantly decreased in Tlr4−/− mice and was due to TLR ligation peripheral to IECs**

As TLR4 is necessary for the innate immune system to respond to LPS (Beutler et al., 2001), we investigated whether Tlr4−/− mice would exhibit LPS-induced small intestinal injury, to exclude the possibility of alternate mechanisms. IE-LPS (10mg/kg) caused negligible change
in villus height in Tlr4−/− mice (Figure 3C) and negligible IEC apoptosis and cell shedding (Figure 3D). However, it should be noted that when Tlr4−/− mice were administered 10mg/kg PE-LPS, although this resulted in negligible change in villus height (Figure 3C), a moderate increase in IEC apoptosis and cell shedding of 7.0±1.0% was seen (Figure 3D). To exclude the possibility that IEC apoptosis and shedding was affected by direct TLR ligation in intestinal epithelial cells, we additionally tested the response of Vil-cre Myd88−/− mice which lack the TLR signaling adapter molecule MyD88 in IECs, to systemic LPS administration. These mice showed very comparable amounts of apoptosis and shedding of 14.1±1.1% compared to WT and heterozygous counterparts (Figure 3E). Furthermore, we tested the small intestinal response to LPS by alternative routes of administration at 1.5h. We found that while intraperitoneal, intravenous, or subcutaneous LPS caused IEC apoptosis and cell shedding, when LPS was delivered directly into the lumen of a ligated segment of small intestine in terminally anaesthetized WT mice, this did not initiate apoptosis and shedding (Figure 3F).

These results suggest that LPS-induced small intestinal injury is dependent on TLR4 signaling peripheral to IECs and that additional bacterial components in PE-LPS cause IEC shedding via TLR4 independent mechanisms.

NfkB1−/− mice were more sensitive, and NfkB2−/− mice more resistant to LPS-induced intestinal injury

NFκB is a major transcriptional regulator downstream of TLR4. We therefore administered PE-LPS to NfkB1−/− and NfkB2−/− mice, to establish whether either of these subunits, integral to the canonical and non-canonical NFκB signaling pathways respectively, is necessary for LPS-induced small intestinal injury. After 10mg/kg LPS, similar villus shortening was seen
in \( \textit{Nfk}b1^{-/-} \) and WT mice (Figure 4A). This genotype also showed similarly increased IEC apoptosis and cell shedding as WT mice (Figure 4B). In contrast, \( \textit{Nfk}b2^{-/-} \) mice showed a significantly attenuated 11% villus height reduction, versus 32% in WT \((P<0.05: \text{Kruskal-Wallis})\) (Figure 4A) and reduced IEC apoptosis and cell shedding (Figure 4B). Interestingly, when administered 0.125mg/kg LPS, \( \textit{Nfk}b1^{-/-} \) mice showed greater villus shortening (Supplementary Figure 2) at 27% \((P<0.05: \text{Kruskal-Wallis})\) compared to 5% in WT mice (Figure 4A), and significantly greater IEC apoptosis and cell shedding at 12.9\%\(\pm\)1.7\% \((P<0.05: \text{Kruskal-Wallis})\) (Figure 4C) compared to WT mice \((5.5\%\pm1.3\%)\). IEC apoptosis and cell shedding in \( \textit{Nfk}b2^{-/-} \) mice administered 0.125mg/kg LPS were negligible \((0.8\%\pm0.2\%; P<0.05: \text{Kruskal-Wallis})\). Together, these results suggest that LPS-induced intestinal injury is dependent on NFκB2, while NFκB1 may be necessary to suppress IEC apoptosis and cell shedding.

**LPS induced a significant increase in small intestinal \textit{Tnf} mRNA**

As activation of Caspase-3 does not categorically confirm that cell death has occurred by apoptosis, we performed an array analysis of 89 genes associated with various cell death pathways in PE-LPS treated compared to untreated WT animals. We found that LPS predominantly altered expression of genes associated with apoptosis, rather than those associated with autophagy or necrosis (Figure 5A). \textit{Tnf} and \textit{Cd40} showed marked up-regulation, and we therefore analyzed these two pro-apoptotic genes by qPCR using triplicate samples from individual animals (Figure 5B). This showed a mean normalized gene expression ratio of +32.0 in \textit{Tnf} mRNA \((P<0.05: \text{randomization test})\). QPCR also showed a non-significant increase of +2.1 in \textit{Cd40}. These data, in conjunction with histopathological
findings and activation of Caspase-3, suggest that apoptosis is the predominant form of cell
death occurring in LPS small intestinal injury.

**TNF caused small intestinal injury equivalent to LPS**

As TNF is a key mediator of endotoxic-shock, and was markedly upregulated at the mRNA
level in our array, we tested whether TNF would cause comparable enteric injury to LPS. At
1.5h, TNF (0.33 mg/kg; i.p.) caused equivalent duodenal villus shortening (Figure 6A) to that
seen with 10mg/kg PE-LPS (268.4±20.9µm and 260.5±15.0µm respectively). Although less
IEC apoptosis and cell shedding were observed with TNF (7.0%±1.0%) compared to
10mg/kg PE-LPS treated mice (12.5%±1.7%)(Figure 6B), this likely reflects a faster small
intestinal response to TNF (Supplementary Figure 3), as it bypasses TNF induction.

These data, together with significant induction of intestinal *Tnf* mRNA, suggest that TNF is
central in the pathogenesis of LPS-induced small intestinal injury.

**Tnfr1<sup>−/−</sup> mice were completely resistant to LPS-induced apoptosis and cell shedding**

We decided to further examine the role of TNF, by testing whether TNF receptors
TNFR1/p55 or TNFR2/p75 were required to cause LPS-induced gut injury. When *Tnfr1<sup>−/−</sup>*
mice were administered 10mg/kg PE-LPS for 1.5h, there was no villus shortening (Figure
6C) and significantly less IEC apoptosis and cell shedding were seen relative to WT animals
(0.1%±0.1%, *P*<0.05;Kruskal-Wallis, Figure 6D). By contrast, the same dose administered to
*Tnfr2<sup>−/−</sup>* mice caused 68% of the response caused in WT, at 8.5±1.0% IEC apoptosis and cell
shedding, although this did not cause a significant change in villus height (Figure 6C). These
findings suggest that TNFR1 signaling is required to drive LPS-induced IEC apoptosis and
cell shedding, with potential enhancement by TNFR2.
Nfκb1−/− mice were highly sensitive, and Nfκb2−/− resistant, to TNF-induced small intestinal injury

NFκB is also a major exponent of downstream TNFR signaling. We therefore administered TNF to Nfkβ1−/− and Nfkβ2−/− mice. Nfκb1−/− mice were highly sensitive to TNF (Supplementary Figure 2) with a significant reduction in villus height (160.1±7.3µm) compared to TNF-treated WT mice (268.4±20.9µm, P<0.05: ANOVA, Figure 6E). This correlated with increased IEC apoptosis and cell shedding at 9.9±0.7% IEC apoptosis and cell shedding in NFκB1−/− versus 7.0±1.0% in WT (P<0.05; ANOVA). Conversely, Nfkβ2−/− mice were resistant to TNF-induced reduction in villus height (340.1±15.1µm) and IEC apoptosis and cell shedding (2.5±0.7%) compared to similarly treated WT animals (both P<0.05; Figure 6F).

These data suggest that IEC apoptosis and cell shedding in response to LPS or TNF, are regulated by a common NFκB signaling pathway; being suppressed by NFκB1, whilst promoted by NFκB2.
Discussion

We present a detailed study of acute LPS induced murine gut injury. Systemic LPS administration caused rapid IEC apoptosis and cell shedding in the murine small intestinal villus, and this resulted in shortening of the villus, fluid effusion into the small intestinal lumen and diarrhea.

We have characterized the dose response and the kinetics of this highly dynamic phenomenon and demonstrate that it occurs within a tightly defined time period. All regions of the small intestine responded in a similar manner to LPS and in all cases apoptosis and cell shedding occurred in the apical 50% of the villus rather than exclusively at the tip. Using knockout mouse models, we confirmed that TLR4 signaling peripheral to the IEC was required, and that TNFR1-mediated signaling was essential for these events, with an NFκB2 dominant response favoring apoptosis.

Although there is an abundance of literature describing small intestinal crypt apoptosis several hours after the induction of endotoxic or septic shock (Cinel et al., 2002; Coopersmith et al., 2003; Guma et al., 2011), we present novel observations that the villus IECs respond much more rapidly than crypt IECs, and exhibit exquisite susceptibility to apoptosis and cell shedding in the earliest stages following LPS administration. The only other study to date that has examined small intestinal villus epithelial shedding in response to LPS, studied this response from 5.5h post LPS administration by in-vivo confocal microscopy; correlating gap formation with gut barrier dysfunction (Lai et al., 2013). This highlights the necessity of a detailed study of the kinetics of this response, as in our model we found that the number of shedding events was profoundly reduced by 4h after LPS administration and the maximum response was observed as early as 1.5h. We found that although multiple organ failure in the
context of endotoxic shock has been extensively investigated, most commonly by biochemical parameters, obvious organ injury in terms of apoptosis was confined to the small intestine at the early time-points examined herein. The reasons underlying this selective early injury to the villus IECs of the murine small intestine are not entirely clear. However, this phenomenon has been attributed to the greater sensitivity of the intestinal epithelium to mitochondrial damage than epithelia found in other commonly injured organ systems such as the lung. Interestingly, in the feline septic shock model in which this was demonstrated, other obvious hemodynamic derangements to which this effect may have been attributed such as hypotension, intestinal hypoperfusion and hypoxia were shown not to be responsible. (Julian et al., 2011). Additionally, in our own studies, this small intestinal injury occurred by 1.5h not only when LPS was adiministered intraperitoneally, but also when given intravenously or subcutaneously, suggesting that this injury is not due to a localized phenomenon.

Clinically, we found that the onset of diarrhea correlated temporally with IEC apoptosis and cell shedding. This suggests that the shedding of epithelial cells permits net movement of fluid from the plasma into the intestinal lumen. This may be directly due to the rapid and uncoordinated shedding of IECs, potentially in conjunction with increased vascular permeability, which causes disruption of tight junctions and the paracellular space. Our own studies have previously shown that barrier loss in the intestine occurs at sites of excessive cell shedding (Kiesslich et al., 2012), and that the direction of fluid movement through epithelial defects is highly dependent on the osmotic and hydrostatic gradients across the epithelium. The concept of acute fluid exudation into the intestinal lumen after the administration of inflammatory stimuli has also been recognized in other studies utilizing LPS or TNF (Gadjeva et al., 2007; Kiesslich et al., 2012). It was not until 5h after LPS administration however, that we found movement of larger molecules (FD4) from the lumen to the plasma.
This is in agreement with findings from in-vivo confocal microscopy that from 5.5h after LPS administration, FD4 entered cell-free gaps and paracellular spaces (Lai et al., 2013).

In our model, a high purity preparation of LPS caused villus IEC apoptosis and cell shedding through a TLR4-dependent mechanism, but phenol-extracted LPS of lower purity was capable of inducing a moderate response via TLR4-independent mechanisms, most likely due to ligation of alternative Toll-like receptors (TLRs) by residual impurities such as bacterial RNA. In support of other PAMPs causing this type of response, another recent study has demonstrated that the apoptosis in the intestinal villus by the viral PAMP, double stranded RNA, occurred via TLR3 (McAllister et al., 2013). TLR3, in contrast to other TLRs, signals exclusively via the TRIF rather than Myd88 pathway (Kawai and Akira, 2011). As such, this agonist represents an unusual type of inflammatory response. It caused apoptosis by a TRIF dependent and TNF independent mechanism, which peaked at 2h post administration, possibly reflecting delayed activation of the TRIF pathway compared to the Myd88 pathway (Pålsson-McDermott and O'Neill, 2004).

Most previous studies have found only low level expression of TLRs, including TLR4 in IECs (Abreu, 2010). Therefore, rather than occurring in IECs themselves, initial recognition of systemically delivered LPS likely occurs via TLR4 ligation in monocytes and macrophages, which in turn rapidly secrete cytokines including TNF (Beutler et al., 1985). To confirm this mechanism in our model, we administered LPS by i.p. injection to Villin-cre MyD88<−/− mice that specifically lacked intestinal TLR signal transduction (Figure 3E). They showed comparable IEC apoptosis and cell shedding to their heterozygous counterparts, and WT mice of the same strain, demonstrating that peripheral TLR signaling is required for LPS-induced small intestinal injury.
LPS signaling is additionally dependent on delivery of LPS to the cell membrane in a bioactive form by lipopolysaccharide binding protein (LBP) and the adapter molecules CD14 (Wright et al., 1990) and MD-2 (Shimazu et al., 1999). TLR4 signaling may also therefore be fundamentally different in IECs, preventing what would be constant stimulation by the luminal Gram negative bacterial population. Indeed, in cell culture of m-ICcl2 murine IECs, TLR4 was found to reside within the Golgi apparatus, rather than at the cell membrane (Hornef et al., 2002). In our studies, we found that LPS (1mg/ml) instilled directly into the lumen of a ligated segment of small intestine for 1.5h did not cause apoptosis and cell shedding. Similarly, double stranded RNA has failed to elicit apoptosis and shedding when administered orally (McAllister et al., 2013).

The rapid increase in plasma TNF concentration after LPS administration has been previously characterized (Copeland et al., 2005). In the current study, we also demonstrated a large fold change in Tnf mRNA abundance in small intestinal epithelial enriched extracts 1.5h after LPS administration. The significance of TNF as the critical mediator in our model was further demonstrated by TNF administration causing very comparable IEC apoptosis and cell shedding to LPS, consistent with previous results (Garside et al., 1993; Piguet et al., 1998). In the intestinal epithelium, and in intestinal cell lines, TNFR1 is expressed to a greater extent than TNFR2 (Lau et al., 2011; Mizoguchi et al., 2002), although the latter can be induced by inflammatory cytokines (Mizoguchi et al., 2002). TNFR1 has well defined pro-apoptotic effects (Locksley et al., 2001), and in our model TNFR1 was essential for LPS induced IEC apoptosis and cell shedding. This suggests that direct TNFR1 ligation occurs at the level of the IEC, although interactions in intermediary cell types, such as endothelial, or myofibroblast cells, cannot be excluded. The role of TNFR2 has been less well characterized and interestingly the intestinal epithelial response to LPS was moderate in Tnfr2−/− mice,
suggesting that this receptor does participate in LPS-induced IEC apoptosis, possibly by enhancement of TNFR1 signals through degradation of TRAF2, resulting in termination of NFκB signaling (Rodríguez et al., 2011).

Mice deficient in NFκB1, an individual NFκB family member which signals via the canonical activation pathway, developed more IEC apoptosis and cell shedding in response to TNF than WT. This is not surprising, since TNFR1, as well as initiating apoptosis, also induces the expression of anti-apoptotic genes via NFκB (Wang et al., 1998). However, an interesting finding to emerge from our study was that mice deficient in NFκB2, an NFκB family member which signals via the alternative activation pathway, were more resistant to villus IEC apoptosis and shedding in response to TNF, compared to WT. This suggests that alternative NFκB2 pathway activation contributes to cell shedding and apoptosis. A possible mechanism to account for this effect could be due to the NFκB2 precursor acting as an inhibitor of NFκB activity (Basak et al., 2007; Fukushima et al., 2012). Absence of NFκB2 may therefore result in an extended expression of p65/RelA induced anti-apoptotic genes (Summarized in Figure 7). The importance of p65/RelA nuclear translocation in IECs that do not undergo shedding has been previously demonstrated in Cryptosporidium parvum infection (Foster et al., 2012). Alternatively, NFκB2 containing dimers may directly terminate the transcription of anti-apoptotic genes or induce the expression of pro-apoptotic genes.

In conclusion, we provide a detailed description of the early events and mechanisms that are responsible for acute, LPS-induced small intestinal injury. LPS therefore represents a robust, rapid, and consistent stimulus for inducing pathological small intestinal epithelial cell apoptosis and shedding. Further study of this phenomenon may be highly relevant to our
understanding of the initial pathogenesis of acute small intestinal disease states and diarrheal illnesses, and the intestinal manifestations of acute systemic disease states such as septic/endotoxic shock. Additionally, the very acute small intestinal lesions documented in this study may also contribute to development of chronic inflammation, such as is observed in IBD. Indeed, defective small intestinal permeability such as may occur in IEC shedding, has been shown to contribute to development of chronic colitis (Arrieta et al., 2009), meaning that this model may have relevance in CD or UC development. Ultimately this may lead to the development of novel therapeutic strategies to ameliorate pathological villus epithelial cell apoptosis, shedding, and gut barrier dysfunction.
Materials and Methods

Animals

Wild-type (WT) C57BL/6 mice supplied by Charles River (Margate, UK), and transgenic strains on a C57BL/6 background, including Nfkb1−/− and Nfkb2−/− mice (Caamaño et al., 1998; Sha et al., 1995) and Tlr4−/− mice (Hoshino et al., 1999) (generated by Shizuo Akira and supplied by Mark Taylor) were maintained at the University of Liverpool. Tnfr1−/− and Tnfr2−/− mice (Peschon et al., 1998) were maintained at the Saban Research Institute at Children’s Hospital Los Angeles. Vil-cre Myd88−/− mice were maintained at the Disease Modeling Unit, University of East Anglia. All procedures were performed on adult mice (minimum age 9 weeks) under appropriate UK Home Office licenses or with approval and monitoring by the Children’s Hospital Los Angeles Institutional Animal Care and Use Committee.

Generation of Vil-cre Myd88−/− mice

Myd88fl/fl that express a truncated mutant MyD88 protein following removal of the floxed region, were bred with heterozygous Villin-cre mice which conditionally express Cre recombinase under control of the villin promoter. Offspring were genotyped for presence of the wild-type Myd88, mutated Myd88, and Cre alleles. Mice were on a C57BL/6 genetic background.

Lipopolysaccharide

LPS from Escherichia coli O111:B4 purified by phenol-extraction (PE-LPS), or ion-exchange chromatography (IE-LPS) (Sigma-Aldrich; Gillingham, UK) was diluted in sterile phosphate-buffered saline (PBS) and administered to mice by intraperitoneal (i.p.) injection.
TNF

Murine recombinant TNF (Peprotech.Ltd; London, UK) was diluted in sterile water and administered by i.p. injection to mice at 0.33mg/kg body weight.

Tissue processing

Following euthanasia, the intestinal tract was dissected en bloc. The intestinal lumina were flushed with PBS and immediately fixed in 10% neutral buffered formalin with selected organ samples. After 24h fixation, tissue was routinely processed and embedded in paraffin wax. Tissue-sections (3-5μm thickness) were prepared and stained either with hematoxylin and eosin (H&E), or used for immunohistochemistry (IHC).

Immunohistochemistry for apoptotic IECs

Tissue sections were treated with 1% hydrogen-peroxide in methanol to block endogenous peroxidases, followed by heat-induced antigen retrieval in 0.01M citrate acid buffer (pH6) and incubation with a rabbit polyclonal anti-active Caspase-3 antibody (AF835:R&D systems; Abingdon, UK). Peroxidase-labeled anti-rabbit EnVision™ (Dako; Cambridge, UK) and 3,3'-diaminobenzidine were used for visualization.

Quantification of active Caspase-3 positive cells

For quantification of apoptotic and shedding intestinal epithelial cells (IECs), individual epithelial cells were counted from the base of the villus (above crypt level) to the mid-point of the villus tip in 18-20 well orientated hemivilli at 400x magnification (delineated by red line in Figure 2B). IECs were categorized according to the following criteria:

- “Normal” if there was no/weak diffuse non-specific brown staining and cells had a basally located basophilic nucleus.
• “Apoptotic” if there was defined positive staining which was confined to cytoplasmic or nuclear borders when compared to any background staining of neighboring IECs.

• “Shedding” if there was defined positive staining which was confined to cytoplasmic or nuclear borders and in addition there was apical elevation of the cytoplasmic membrane, and/or an apically positioned nucleus.

Crypt IECs were counted from the crypt base to the crypt-villus junction in 19-20 well orientated duodenal hemicrypts per mouse. Crypt IECs were simply categorized as “normal” or “apoptotic” as no discernable evidence of shedding was observed within crypts.

Cell Positional Data

To allow comparison of cell positional data, villi were adjusted to a fixed length of 100 cells by using Wincrypts® software (Cancer Research Campaign 1999). Data are then represented as percentage of villus length, 0% therefore representing the villus base, and 100% representing the villus tip.

Measurement of Villus Height

Image J (Schneider et al., 2012) was used to assess images captured by a Leica DMLA microscope, by setting the scale with a hemocytometer at 100x magnification. All images were captured at 100x magnification, and villi were measured by using the segmented line tool. Each segmented line was placed to originate at the base of the villus, above the level of adjoining crypts, and a segmented line extended to the villus tip, following any curvature of the villus. The mean of these segmented line lengths for 10 well-orientated villi was calculated for each animal, and a mean value was then calculated for each group.
**Gut Permeability Assessment**

Fluorescein-isothiocyanate-conjugated dextran (FD4: Sigma-Aldrich, Gillingham, UK) was diluted to 22mg/ml in PBS and administered at 20ml/kg by oral gavage ± i.p. LPS. Plasma fluorescence was measured by TECAN Infinite® F200 plate reader (excitation 485nm, emission 535nm) from blood collected post-mortem, 5h after gavage in order to allow FD4 to be distributed throughout the intestinal tract, prior to them being euthanized via a rising CO₂ concentration, and blood taken by cardiac puncture. To assess the effect of LPS on permeability, it was administered at set time points prior to the end of the experiment, i.e. at 1.5h, 3h, and at the same time as FD4 for the 5h time point. Plasma was separated from heparinized whole blood by centrifugation at 5000 rpm for 2 minutes in a minicentrifuge.

**Real-time PCR**

Small intestinal extracts were isolated with chelation buffer solution as previously described (Flint et al., 1991), and RNA was isolated with a High pure RNA Tissue Kit (Roche; Burgess Hill, UK). Reverse-transcription was performed with a RT² reverse-transcription kit (SABiosciences; Crawley, UK). An 89 gene Cell-Death Pathway Finder array (SABiosciences) was performed on a Roche LightCycler®480 followed by validation with replicate samples using TaqMan® gene expression assays for β-actin (Mm01205647_g1), TNF (Mm00443260_g1) and CD40 (Mm00441891_m1; Life Technologies; Paisley, UK). Cycling conditions were performed as per manufacturer instructions.
Data analysis

Data represent mean±SEM. Comparisons were made between treatment groups and controls using SigmaPlot 12© (Systat Software; London, UK). Normally distributed data were assessed by ANOVA with Holm-Sidak post-hoc test, and non-parametric data were analysed by ANOVA on ranks (Kruskal-Wallis) with Dunn’s post-hoc test. \( P<0.05 \) was considered significant. REST© software was used for comparison of qPCR data from individualized samples by randomization test as previously described (Pfaffl et al., 2002). N numbers indicate the total number of mice studied.
Acknowledgements

The authors would like to thank David Berry (University of Liverpool) for technical assistance, Mark Taylor and Alice Halliday (The Molecular and Biochemical Parasitology Group, Liverpool School of Tropical Medicine), Professor Shizuo Akira, and Associate Professor Satoshi Uematsu (Department of Host Defense, Research Institute for Microbial Diseases Osaka University, Japan) for the provision and use of Tlr4−/− mice. The authors also thank Simon Clare and Gordon Dougan, Wellcome Trust Sanger Institute, Cambridge, for the Villin-Cre Myd88−/− mouse strain.
Competing Interests Statement

Authors’ disclosures of any potential conflict of interest are as follows:

JMW: No disclosures
CAD: No disclosures
AJMW: No disclosures
MRF: No disclosures
JCM: No disclosures
MDB: No disclosures
RS: No disclosures
KRH: No disclosures
LJH: No disclosures
JHC: No disclosures
BJC: No disclosures
DMP: No disclosures
Author Contributions

Project development. Composition of manuscript.

CAD: Original conceptualization of project. Animal and laboratory procedures. Data

AJMW: Original conceptualization of project. Grant and manuscript preparation.


RS: Manuscript preparation.


BJC: Original conceptualization of project. Data analysis. Grant and manuscript preparation.

DMP: Original conceptualization of project. Data analysis. Grant and manuscript preparation.
**Funding**

JMW’s PhD is funded by the Centre for Integrative Mammalian Biology and has also been supported by the *Journal of Comparative Pathology* Educational Trust. MRF is supported by National Institutes of Health Grants K01DK077956 and R03DK090295 and a Senior Research Award from the Crohn's and Colitis Foundation of America. MDB is funded by Wellcome Trust Research Training Fellowship 083823/Z/07/Z. JHC was supported by the EU FP7 Integrated Project INFLACARE. This work was supported in part by a Biomedical Research Unit award from the National Institute for Health Research. KRH gratefully acknowledges the support of the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme grant for Gut Health and Food Safety BB/J004529/1. DMP and BJC are supported by SysmedIBD which is funded by the European Commission within the 7th Framework Programme. AJMW and DMP are supported by Wellcome Trust grant WT0087768MA. AJMW is also supported by BBSRC grant BB/J004529/1.
References


Figure legends

**Figure 1.** LPS at 10mg/kg caused acute diarrhea, villus epithelial cell loss, villus shortening, and increased gut permeability. (A) Small intestine of untreated control (white arrow) and 1.5h LPS treated WT mouse exhibiting fluid filled small intestine devoid of digesta (blue arrow) with diarrheic feces at anus (blue arrowhead). Caeca indicated by white arrowheads. H&E stained sections of duodenum (100x) showing normal villi in untreated control and shortened, blunted and clubbed villi with shed IECs in lumen at 1.5h post-LPS. Bars=200µm. Villus tips (630x) in untreated control and shedding IECs at 1.5h post-LPS (arrows). Bars=25µm. (B) Villus heights for duodenum, jejunum, and ileum (n=6). (C) Hemivillus IEC counts from base to apex for duodenum, jejunum, and ileum (n=6). (D) Plasma fluorescence measured after 10mg/kg PE-LPS and gavage of FD4. (N=6, 1 outlier excluded at 1.5hr). *=P<0.05, **=P<0.01. Comparisons by ANOVA in A, and Kruskal-Wallis in B and C.

**Figure 2.** LPS caused activation of Caspase-3 with concomitant apoptosis and cell shedding of villus IECs and relatively spared the crypts. (A) Duodenal sections labeled for active Caspase-3 by IHC. Arrowhead indicates positively labeled apoptotic IEC with unaltered morphology, and arrow indicates positively labeled IEC with shedding morphology (50x bars=200µm, 630x=25µm). (B) Example of a duodenal villus 1.5h following 10mg/kg PE-LPS administration to a C57BL/6 female mouse. Individual cells were counted along the epithelial monolayer lining one side of a villus (delineated by red line) from base to tip, and therefore referred to as a “hemivillus”. Cells were categorized as “normal”, “apoptotic”, or “shedding”. Eighteen to twenty hemivilli were analysed for each intestinal segment for each
individual animal. (C) Quantification of apoptotic and shedding IECs in duodenum, jejunum, and ileum and cell positional quantification of “apoptotic” or “shedding” IECs in duodenum, jejunum, and ileum along villus length (0 represents villus base, 100% represents villus tip). (D) Quantification of active Caspase-3 positive cells in villus versus crypt IECs. (E) Villus versus crypt IEC counts. N=6 female mice/group,*=P<0.05, comparisons by ANOVA.

**Figure 3. LPS caused a plateau in apoptosis and shedding at a threshold dose through TLR4 signaling peripheral to epithelial cells.** Heights of duodenal villi, 1.5h after PE-LPS administration at indicated doses (A) and quantification of percentage apoptotic and shedding IECs by assessment of duodenal sections labeled for active Caspase-3 by IHC (B). N=6 female mice/group for PBS, 0.125mg/kg and 10mg/kg, and n=4 female mice for all other groups. (C) Villus heights in WT or Tlr4−/− mice 1.5h after phenol-extracted (PE-LPS) or ion-exchange chromatography extracted (IE-LPS) at 10mg/kg, and quantification of IEC apoptosis and cell shedding in IHC labeled duodenal sections (D) (n=6). (E) Quantification of apoptotic and shedding IECs in WT (n=6), Villin-cre MyD88−/− (n=3) and Villin-cre MyD88+/− (n=3) mice by assessment of duodenal sections labeled for active Caspase-3 by IHC. (F) Small intestinal villi from WT female mice 1.5h post PE-LPS by subcutaneous (s.c.) or intravenous (i.v.) routes exhibited villus IEC apoptosis and shedding except when LPS was instilled intraluminally (active Caspase-3 IHC, duodenum shown for s.c. and i.v. LPS (n=3 per group), ileum shown for intraluminal administration instilled with 1mg/ml LPS (n=4 per group) bars=25µm).*=P<0.05, **P< 0.01. Comparisons by ANOVA in A, B, and C, and by Kruskal-Wallis and pairwise comparison of LPS treated mice only in D.
Figure 4. Nfkβ1−/− mice were more sensitive, and Nfkβ2−/− mice more resistant to LPS-induced small intestinal injury. Villus heights of duodenal villi 1.5h after 0.125mg/kg or 10mg/kg PE-LPS in WT, Nfkβ1−/−, and Nfkβ2−/− mice (comparisons between genotypes within same dosage groups only)(A). Quantification of apoptotic and shedding IECs in duodenal sections labeled for active Caspase-3, in WT, Nfkβ1−/− and Nfkβ2−/− mice, 1.5h after 10mg/kg PE-LPS (B) and after 0.125mg/kg PE-LPS (C). N=12-14 (male and female equally represented), *=P<0.05, comparisons by Kruskal-Wallis.

Figure 5. LPS induced a significant increase in small intestinal Tnf mRNA. (A) QPCR array data for selected genes that exhibited ≥2-fold expression ratios out of 89 cell-death pathway associated genes assessed in pooled epithelial enriched extracts (B) Mean gene expression ratio of PE-LPS treated (10mg/kg, 1.5h) versus untreated WT female mice for Cd40 and Tnf. N=4, P<0.05: randomization test.

Figure 6. LPS induced small intestinal IEC apoptosis and cell shedding via TNF and TNFR1, and was regulated by NFκB. Villus heights and IEC apoptosis and shedding 1.5h after 10mg/kg PE-LPS or 0.33mg/kg TNF in WT mice (A, B), after 10mg/kg PE-LPS in WT, Tnfr1−/−, and Tnfr2−/− mice (C, D) and after TNF administration in WT, Nfkβ1−/− and Nfkβ2−/− mice (E, F), *=P<0.05. N=4-6 mice per group, comparisons by ANOVA, except in D (Kruskal-Wallis).

Figure 7. Diagram summarizing putative mechanism by which LPS induces apoptosis in IECs. Systemically delivered LPS is first recognized predominantly by resident TLR4
expressing mononuclear cells (monocytes/macrophages/dendritic cells) which produce TNF. TNF is released into the systemic circulation and binds with TNFR1 on intestinal epithelial cells, triggering IEC apoptosis and cell shedding if NFκB2 signaling dominates, or cell survival if NFκB1 signaling dominates.
Translational Impact

Clinical issue

Epithelial cell loss and a defective barrier likely represents the initial injury in a number of acute intestinal and diarrheal diseases, and has also been implicated in the pathogenesis of chronic intestinal inflammatory diseases, such as Crohn’s disease and ulcerative colitis. Both these conditions have been associated with increased numbers of intestinal epithelial cell shedding events with increased gut permeability. However, the molecular regulation of intestinal epithelial cell shedding and intestinal barrier function during inflammatory disease states remains poorly understood, hence a robust animal model would greatly facilitate in depth investigation of this process. Mice exhibit a morphologically analogous form of small intestinal villus epithelial cell shedding to that observed in humans and also provide for greater mechanistic insight because of an increasing number of established transgenic and conditional mutant models. Utilizing the inflammatory response induced by systemic administration of lipopolysaccharide (LPS) in mice, we have therefore developed a reliable model for investigating the molecular events determining intestinal epithelial cell shedding.

Results

A threshold dose of $\geq 0.125$mg/kg LPS delivered by intraperitoneal injection induced rapid and dynamic villus IEC apoptosis and shedding with concomitant activation of Caspase-3 peaking at 1.5h post administration. This coincided with significant villus shortening, fluid exudation into the gut and the onset of diarrhea. Almost universal concomitant activation of Caspase-3 in shedding cells suggests that apoptosis is triggered within the epithelium prior to cells being shed. Through examination of responses to LPS in transgenic mice, we found that
TNFR1 was essential for induction apoptosis and shedding. Furthermore, the fate of the IEC was dependent on NFκB, with signaling by NFκB1 favoring cell survival, and signaling via NFκB2 favoring apoptosis.

**Implications and future directions**

Systemic LPS administration in mice represents a robust, consistent, and reliable stimulus to study pathological intestinal epithelial cell shedding to parallel that observed in acute human intestinal diseases. These data also suggest that villus epithelial apoptosis and cell shedding can cause acute fluid exudation into the gut leading to diarrhea. Through detailed examination of the kinetics, dose response and signaling mechanisms underlying this process, we have developed a valuable model which will enable further investigation of epithelial gap formation and barrier dysfunction in the inflamed intestinal epithelium and likely support development of better targeted treatments for inflammatory disorders.
Expression ratio in LPS treated versus untreated WT mice

Pro-apoptotic
Anti-apoptotic
Necrosis associated

A

B

Expression ratio in LPS treated versus untreated WT mice

Cd40
Tnf

*
Small intestinal epithelium

Shedding

Apoptosis

Caspase 3

Cleaved Caspase 3

Anti-apoptotic genes

NFkB1

NFkB2

TNF

TNF receptor 1 (TNFR1)

LPS

TLR4

Myd88

NFkB

TNF

Tnf

Resident tissue macrophages

Disease Models & Mechanisms

DMM

Accepted manuscript