Absence of Fer protein tyrosine kinase exacerbates endotoxin induced intestinal epithelial barrier dysfunction in vivo

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**Background and aims:** Fer kinase is activated by a number of growth factors and cytokines, and phosphorylates cortactin during cell shape change induced cortical actin reorganisation. In addition, Fer participates in cytoskeletal interactions mediated by cadherins, platelet endothelial cell adhesion molecule 1 (PECAM-1), and integrins, and has recently been implicated in limiting the innate immune response. Here we examined the role of Fer in modulating leucocyte recruitment and epithelial barrier function in the gut in response to lipopolysaccharide (LPS).

**Methods:** Mice targeted with a kinase inactivating mutation (FerDR) or strain matched wild-type (129Sv/J) mice were studied after intraperitoneal injection of LPS. Intravital microscopy was used to examine intestinal leucocyte kinetics, and leucocyte infiltration was assessed by fluorescence activated cell sorting. Systemic inflammation was assessed by measuring lung myeloperoxidase activity. Epithelial barrier function was assessed in vivo using blood to lumen $^{51}$Cr-EDTA clearance, with or without antibody based depletion of circulating neutrophils.

**Results:** LPS induced a significant increase in leucocyte adhesion and neutrophil infiltration into the intestinal tissue, and increased blood to lumen $^{51}$Cr-EDTA clearance. Pretreatment with neutrophil depleting antibody completely abrogated this response in wild-type mice. In FerDR mice, LPS induced leucocyte adhesion within the intestinal venules was exacerbated and associated with a trend towards increased neutrophil transmigration relative to wild-type mice. Surprisingly, LPS induced epithelial barrier permeability was increased 2.5-fold in FerDR mice relative to wild-type mice, and this barrier defect was only partly attenuated by depleting circulating neutrophils by $\geq 93\%$.

**Conclusions:** Fer plays a role in regulating LPS induced epithelial barrier dysfunction in vivo through both neutrophil dependent and neutrophil independent mechanisms.

**Abbreviations:** LPS, lipopolysaccharide; PTK, protein tyrosine kinase; MPO, myeloperoxidase; Fer, fes related; FerDR, mice homozygous for Fer deficient; LD$_{50}$, lethal dose that kills 50% of group; PE, phycoerythrin

Lipopolysaccharide (LPS) or endotoxin (a component of the outer membrane of Gram negative bacteria) is a dominant bacterial antigen which is capable of activating a variety of cell types to release inflammatory mediators, induce leucocyte infiltration, and cause mucosal dysfunction. Infections involving LPS continue to be a major clinical problem resulting in complications such as multiple organ dysfunction, septic shock, and mortality. Neutrophil recruitment from the microcirculation is a hallmark feature of the host's normal response to infection. However, in the gut, neutrophil recruitment has been directly associated with tissue damage and epithelial barrier dysfunction in response to various stimuli. Therefore, regulation of leucocyte recruitment and epithelial barrier integrity in response to toxins such as LPS, is essential in maintaining homeostasis in the gut.

Fer (Fes related) is a ubiquitously expressed cytoplasmic protein tyrosine kinase (PTK) belonging to the group IV subfamily of PTKs, which also includes the closely related Fps/Fes PTK. Fer kinase is activated by a number of extracellular stimuli, including epidermal and platelet derived growth factor, insulin, erythropoietin, stem cell factor, collagen and thrombin and on aggregation of the high affinity IgE receptor FcεRI. Fer is also activated by cell shape changes, leading to phosphorylation of cortactin, an important regulator of the cortical actin cytoskeleton. Fer has been implicated in the regulation of adherens junctions and crosstalk to focal adhesions. At adherens junctions, Fer and the tyrosine phosphatase, phosphotyrosine phosphatase 1B, cooperatively regulate the tyrosine phosphorylation status of cadherin bound β-catenin, which plays a key role in connecting adherens junctions to the cytoskeleton. At focal adhesions, Fer regulates the phosphorylation status of the scaffolding proteins p130Cas and p120 catenin. Overexpression of Fer in fibroblasts diminished integrin based cell adhesion while Fer deficient mast cells displayed increased adhesiveness and reduced motility in response to FcεRI engagement. These Fer deficient mast cell phenotypes correlated with diminished p38 activation. Fer has also been localised with newly assembling microtubules in protrusions at the leading edge of motile endothelial cells and was implicated in the phosphorylation of the cell-cell adhesion molecule platelet endothelial cell adhesion molecule 1 (PECAM-1). Fer mediated phosphorylation of PECAM-1 may play an important role in regulating signalling in vascular endothelial cells or diapedesis of activated leukocytes. PECAM-1 deficient mice display an enhanced innate immune response to LPS challenge. Interestingly, we have recently reported that Fer deficient mice display enhanced recruitment of leukocytes to skeletal muscle in response to LPS challenge.
Taken together, these studies suggest key roles for Fer in growth factor and cytokine signalling, cell-cell and cell-matrix interactions, cell migration, and cytoskeletal remodelling. All of these cellular functions are important in the innate immune response. To date, the role of Fer kinase in the innate immune response in the gastrointestinal tract is not known. In this study, we evaluated the in vivo role of Fer in gastrointestinal inflammation induced by LPS. Fer deficient mice displayed an enhanced inflammatory response to LPS which correlated with increased leucocyte adhesion to endothelial cells in the gut microvasculature, and a striking neutrophil dependent epithelial barrier dysfunction. These observations provide compelling new evidence for a physiological role for Fer in regulating innate immunity in the gastrointestinal system.

**MATERIALS AND METHODS**

**Animals**

Fer kinase was inactivated in mice by targeting the fer locus with a kinase inactivating missense mutation (Fer<sup>D743R</sup>), as previously described. In addition to abolishing kinase activity, the D743R mutation was found to destabilise the Fer protein, leading to its rapid degradation. Mice homozygous for the Fer<sup>D743R</sup> mutation therefore behave essentially as Fer null animals. Fer<sup>DR</sup> mice (maintained in a 129Sv/J background) were rederived by Jackson Laboratories and a mutagenesis program. Fer<sup>DR</sup> mice (maintained in a 129Sv/J background) were rederived by Jackson Laboratories and a mutagenesis program. Fer<sup>DR</sup> mice (maintained in a 129Sv/J background) were rederived by Jackson Laboratories and a mutagenesis program.

**Systemic LPS administration**

LPS (0.5 mg/kg) from *Escherichia coli* 0111:B4 (Biosciences Inc., La Jolla, California, USA), containing less than 0.8% contaminating bacterial proteins, was administered intraperitoneally. This dose of LPS has been previously used to examine leucocyte-endothelial interactions in vivo and is approximately 1/50 of the LD<sub>50</sub> (lethal dose that kills 50% of the group) for mice. Small intestinal intravascular leucocyte kinetics, epithelial barrier integrity, and leucocyte migration in wild-type or Fer<sup>DR</sup> mice were performed 3.5–4.5 hours post intraperitoneal injection of LPS or vehicle as described below. In separate experiments, mice were treated with LPS doses of 0.005, 0.05, 0.5, or 5 mg/kg and leucocyte kinetics were observed.

**Neutrophil depletion**

Mice were depleted of circulating neutrophils by a single intraperitoneal injection of 150 μg of monoclonal antineutrophil antibody RB6 8C5 (Anti-Ly6G; BD Biosciences Pharmingen, Mississauga, Ontario, Canada) 24 hours prior to analysis. Manual counts (performed as described below) showed that circulating neutrophils were depleted by >93% within 24 hours in both wild-type and Fer<sup>DR</sup> mice. For depletion of tissue neutrophils, three injections of 150 μg of RB6 8C5 were given at 24 hour intervals, and mice were analysed 24 hours after the final dose. This treatment regimen is sufficient to deplete resident and circulating neutrophil populations.

**Circulating leucocyte counts**

Whole blood was obtained via cardiac puncture and total leucocyte counts were performed using a Bright-line haemacytometer (Hausser Scientific, Horsham, PA). Leucocyte differential counts were determined by examination of blood smears stained using harleco hemacolour kit (EM Science Gibbstown, New Jersey, USA).

**Intravital microscopy**

Small intestinal microcirculation was examined by intravital microscopy using a previously described method with slight modifications. Briefly, mice were anaesthetised and maintained on inhalation anaesthetic (isoflurane; Bimeda-MTC, Cambridge, Ontario, Canada). A jugular cannulation was performed for administration of rhodamine 6G and body temperature was maintained at 37°C. A segment of small intestine was draped over a viewing pedestal and the submucosal vessels of the terminal ileum were observed using a microscope (Mikron Instruments, Inc., California, USA) with a 20× objective lens (Zeiss Achroplan 20×/0.5W) and a periplan 10× eyepiece (final magnification 1500×). Leucocytes were stained in vivo by intravenous injection of 0.05% rhodamine 6G (Sigma-Aldrich Co., Missouri, USA). Images of the microcirculatory bed were recorded using a video camera (Pieper GMBH, Berlin, Germany) and a video recorder (Panasonic AG-1980 P; Matsushita Electric Ind. Co, Ltd, Osaka, Japan) over a 30 minute time frame. Five post capillary venules (diameter 25–45 μm) were selected in each mouse for study. Rolling leucocytes were defined as those white blood cells moving at a velocity less than that of blood flow in the same vessel. Leucocyte rolling velocity was determined as the time required for a leucocyte to traverse 100 μm of venule, and averaged for 20 leucocytes (μm/s).

Leucocyte flux was calculated as the number of rolling cells crossing a given point per minute (cells/minute). Leucocyte adhesion was quantified as the number of leucocytes that
where $cpm_p$ is counts/min/ml in perfusate, $pr$ is perfusion rate in ml/min, $cpm_{pl}$ is counts/min/ml in plasma, and $wt$ is weight of the intestinal segment in grams.

**In vivo epithelial barrier function**

Epithelial barrier function was quantified by measuring blood to lumen 51-chromium ethylenediamine tetra-acetic acid ($^{51}$Cr-EDTA) movement in the mouse small intestine, as previously described. Briefly, mice were anaesthetised by intraperitoneal injection with a cocktail of 10 mg/kg xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Rogar/STB Inc., Montreal, Quebec, Canada). A jugular cannulation was performed to administer $^{51}$Cr-EDTA and the anaesthetic, as needed, and both renal pedicles were ligated to prevent excretion of $^{51}$Cr-EDTA into urine. A loop of small intestine was fitted with inflow and outflow tubes, and continuously perfused with warmed Tyrodes solution (0.25 ml/min). Samples of luminal perfusate were collected over 10 minute intervals for 60 minutes and $^{51}$Cr-EDTA activity was determined in plasma and luminal perfusate using an LKB Computer Gamma spectrometer. Mucosal permeability was calculated as the plasma to lumen clearance of $^{51}$Cr-EDTA using the following calculation:

$$\text{Clearance} = \frac{cpm_p \times pr}{\times w} \times \frac{cpm_{pl}}{w} \times 100 \text{ g}$$

where $cpm_p$ is counts/min/ml in perfusate, $pr$ is perfusion rate in ml/min, $cpm_{pl}$ is counts/min/ml in plasma, and $wt$ is weight of the intestinal segment in grams.

**Lung myeloperoxidase activity**

Samples of lung tissue were weighed, frozen on dry ice, and stored at $-20^\circ C$ for no more than one week before analysis. Myeloperoxidase (MPO) activity was determined as previously described. One unit of MPO activity was defined as that degrading 1 micromole of hydrogen peroxide per minute at 25°C and expressed as units of MPO activity per milligram of tissue (U/mg tissue).

**FACS analysis of intestinal leucocytes**

Leucocyte populations were quantified by fluorescence activated cell sorting (FACS) analysis by a modified version of a previously described method. Briefly, mice were anaesthetised by intraperitoneal injection of a xylazine/ketamine cocktail, and the vasculature flushed with cold phosphate buffered saline via transcardiac perfusion. The entire small intestine was excised and digested with collagenase type III (Sigma-Aldrich, Missouri, USA), and leucocytes from the lamina propria were isolated on Percoll gradients. Cells were stained with 0.5 µg of fluorescein isothiocyanate labelled anti-Ly-6G (BD Biosciences Pharmingen), 0.2 µg of phycoerythrin (PE) labelled anti-CD3 (BD Biosciences Pharmingen), or 0.2 µg PE anti-F4/80 monoclonal antibodies (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) for neutrophil, lymphocyte, and monocyte populations, respectively. Two colour stained cell populations were analysed by a FACSscan fluorescence analyser (BD, Mountain View, California, USA). Cell viability was assessed using Trypan blue exclusion, and was determined as >90% for all isolated cell populations.

**Statistical analysis**

Data were expressed as the mean (SEM). Groups of data were compared using a non-parametric Mann-Whitney U test or Kruskal-Wallis ANOVA, followed by a Dunn’s multiple comparisons post test.
RESULTS
Systemic response to intraperitoneal LPS challenge in wild-type and FerDR mice
To address the potential role of Fer in regulating the inflammatory response, we first assessed the systemic response observed after intraperitoneal LPS challenge. Total circulating white blood cell and differential analysis were similar in healthy wild-type and FerDR mice, as previously reported. Four hours after intraperitoneal LPS, a systemic response was observed in wild-type mice, as illustrated in fig 1. LPS challenge (0.5 mg/kg) induced a significant decrease in total circulating leukocyte counts in both wild-type and FerDR mice (fig 1A). The drop in circulating leukocyte counts could be accounted for in part by increased accumulation of neutrophils in the lung, as indicated by increased lung MPO activity (fig 1B). Interestingly, a significantly greater LPS induced increase in lung MPO activity was observed in FerDR compared with wild-type mice (fig 1B), suggesting more robust recruitment of granulocytes.

LPS induced leucocyte adhesion is enhanced in the absence of Fer kinase
We next examined the behaviour of leucocytes within the submucosal venules of the small intestine using intravital fluorescence microscopy. In wild-type mice, LPS challenge induced a significant reduction in the number and velocity of rolling cells due to leucocyte sequestration in the lung and upregulation of adhesion molecules on endothelial cells, respectively. A concomitant significant increase in leucocyte adhesion was observed (fig 2). In FerDR mice, similar reductions in leucocyte rolling flux and velocity, and increased adhesion were observed after LPS challenge. Interestingly, the LPS induced increase in leucocyte adhesion was significantly greater in FerDR mice (30%; *p<0.05) compared with wild-type animals. LPS induced leucocyte recruitment was shown to be dose dependent in both wild-type and FerDR mice; however, FerDR mice were two orders of magnitude more sensitive, requiring only 0.05 mg/kg to achieve a maximal response, compared with 5 mg/kg in wild-type mice (fig 3). At 5 mg/kg, no significant difference in leucocyte rolling velocity or adhesion was observed between wild-type and FerDR mice. These data show that loss of Fer kinase activity correlates with increased association and adherence of leucocytes to the endothelial surfaces of intestinal venules following LPS challenge.

LPS induced leucocyte migration in the small intestine
We next examined the number of extravasated neutrophils in the small intestine in response to LPS challenge (fig 4). Wild-type mice had a significant increase in Ly-6 positive cells four hours post LPS challenge, suggesting a significant increase in neutrophil extravasation. Similar results were observed in FerDR mice, with a trend towards increased LPS induced neutrophil recruitment relative to wild-type mice. Small intestine MPO activities were lower than those observed in the lung (fig 1B), and significant increases after LPS treatment could not be determined (data not shown). This suggested that LPS induced neutrophil migration to the intestine was lower than to the lung. Lymphocyte numbers (CD3+ cells) were also significantly increased in wild-type mice after LPS challenge (table 1).

Although a similar trend was observed in FerDR mice, the LPS induced increase did not reach statistical significance. No significant increase in monocyte/macrophage numbers (F4/80 positive cells) were observed after LPS challenge in either wild-type or FerDR mice (table 1), suggesting monocytes were not recruited to the intestine.
LPS induced small intestinal epithelial barrier dysfunction is exacerbated in Fer deficient mice

We next examined LPS induced changes in epithelial barrier integrity by measuring blood to lumen $^{51}$Cr-EDTA clearance for a one hour period, starting 3.5 hours after injection of LPS or saline (fig 5). In saline injected mice, blood to lumen clearance was maintained at an average of 0.3 ml/min/100 g in both wild-type and FerDR mice. LPS challenge induced a significant (2.5-fold) increase in epithelial barrier dysfunction in wild-type mice 3.5 hours post challenge which was maintained for the duration of the experiment (one hour). Remarkably, a 7–10-fold increase in barrier dysfunction was seen in LPS challenged FerDR mice over the same period of time, which was significantly greater than LPS induced epithelial dysfunction in wild-type mice.

Histological analysis of the small intestine was undertaken and scores assigned according to the degree of cellular infiltration (0, 1, or 2; none, mild, or moderate) and the presence or absence (0 or 1) of goblet cell depletion, areas of sloughed epithelium, or Paneth cell depletion. In wild-type mice, LPS (0.5 mg/kg) administration correlated with a mild inflammatory response (fig 6C; compare with fig 6B, saline treated FerDR). Generally, goblet cells and Paneth cells were depleted of their contents and there were areas denuded of epithelium, giving a mean histological score of 3.0 (0.5) ($n = 7$).

Role of infiltrating neutrophils in LPS induced epithelial barrier dysfunction

We next asked if neutrophils are required for LPS induced epithelial dysfunction in the mouse small intestine. This was achieved by depleting circulating neutrophils by pretreating mice with 150 mg of antineutrophil antibody RB68C5 24 hours prior to LPS or vehicle challenge. In our hands, this treatment regimen depleted circulating neutrophils by 93% in both wild-type and FerDR mice. In wild-type mice, neutrophil depletion completely abrogated LPS induced epithelial dysfunction (fig 7). $^{51}$Cr-EDTA clearance levels in LPS challenged neutrophil depleted wild-type mice were not significantly different from saline challenged controls, indicated by the broken line in fig 7. These data demonstrate that in wild-type mice, LPS induced epithelial barrier dysfunction is mediated completely through infiltrating neutrophils. In FerDR mice, similar depletion of circulating...
Neutrophil depletion completely abrogates lipopolysaccharide (LPS) induced epithelial barrier dysfunction in wild-type mice but only partially in Fer deficient mice. Epithelial barrier permeability, represented by $^{51}$-chromium ethylenediamine tetra-acetic acid ($^{51}$Cr-EDTA) blood to lumen clearance, in wild-type mice and FerDR mice. Data were determined.

In this study, LPS challenge increased $^{51}$Cr-EDTA clearance significantly (2.5-fold) by four hours post challenge with LPS 0.5 mg/kg intraperitoneally. In separate groups, mice were treated with RB68C5 to deplete circulating neutrophils. Broken line indicates baseline permeability (saline treated, FerDR, or wild-type mice). Data are expressed as mean (SEM); n = 3. *$p$<0.05, significant increase from saline treated FerDR or wild-type mice; †$p$<0.05 significant increase from RB68C5 treated wild-type mice.

**DISCUSSION**

In this study we have described, for the first time, an important physiological role for Fer kinase in the innate immune response in the gut. We have demonstrated that in response to systemic LPS challenge, leucocyte recruitment to the lung and intestine is enhanced in the absence of Fer kinase and is associated with exacerbated epithelial barrier dysfunction. LPS induced epithelial barrier dysfunction was completely abolished by depleting circulating neutrophils in wild-type mice but was only partially attenuated by neutrophil depletion in FerDR mice. Our data provide compelling evidence for an important physiological role for Fer in regulating intestinal epithelial barrier integrity in response to LPS challenge, and suggest its involvement in both neutrophil dependent and neutrophil independent mechanisms.

In this study, neutrophil accumulation in the lung following systemic challenge was exacerbated in FerDR mice. This may suggest that Fer kinase activity plays a role in neutrophil rigidity/deformity as it has previously been suggested that LPS induced neutrophil sequestration in the lung is due, in part, to increased neutrophil stiffness mediated by F-actin reorganisation. In addition, our data suggest that Fer in wild-type mice may play a role in inhibiting leucocyte adherence on the endothelial surface of intestinal venules. However, significant enhancement in leucocyte migration into intestinal tissue was not observed in FerDR mice although there was a trend in that direction for both neutrophils and lymphocytes. This is in contrast with the massive leucocyte migration observed in response to local LPS challenge in muscle. Recruitment in this present study was most likely muted due to systemic activation recruiting large numbers of neutrophils to the lungs. In addition, our preliminary data using an in vitro cell migration assay has demonstrated that neutrophil chemotaxis towards formylmethionylleucylphenylalanine is enhanced in the absence of Fer kinase activity and would support a role for Fer within the neutrophil in cell movement (data not shown). Taken together these data strongly suggest that Fer acts within neutrophils to influence cytoskeletal changes and cell movement.

An additional role for Fer PTK within the endothelial cells cannot be discounted. For example, Fer may be involved in expression of one or more adhesion molecules known to be involved in rolling (E and P selectin) and adhesion (intercellular adhesion molecule 1 and vascular cell adhesion molecule 1), which are increased in the intestinal microvasculature after systemic LPS. Furthermore, a role in endothelial cells that regulates leucocyte transmigration can be inferred from recent evidence that Fer can phosphorylate PECAM-1, and may therefore be involved in downstream signalling leading to cytoskeletal rearrangements. PECAM-1 functions as an adhesive and signalling molecule known to play a prominent role in the transmigration of leucocytes across endothelium. Our previous work demonstrated that endothelial barrier permeability remained intact in FerDR mice after antigen challenge in sensitised mice, suggesting that the role of Fer within the endothelium is stimulus specific. The role of PECAM-1 phosphorylation by Fer in regulating leucocyte transmigration remains to be determined.

In this study, LPS challenge increased $^{51}$Cr-EDTA clearance significantly (2.5-fold) by four hours in wild-type mice, and was associated with a mild inflammatory response in the tissue, as illustrated by histological observations. This dose of LPS is significantly lower than the LD$_{50}$ and these mice would in all likelihood recover from this challenge. In contrast, the same challenge in FerDR mice induced a 7–10-fold increase in barrier dysfunction which was notably more severe histologically. Similar fold increases have previously been reported in a model of experimental colitis.

It is important to note that epithelial barrier integrity was intact in FerDR mice under control conditions, and exacerbated barrier dysfunction was noted only after LPS challenge. Neutrophils are key leucocytes recruited during the innate immune response and have previously been shown to cause epithelial barrier dysfunction in other models of intestinal inflammation. Therefore, we investigated whether Fer was acting within neutrophils to induce the exacerbated epithelial barrier dysfunction. Using a monoclonal antibody to deplete circulating neutrophils, we completely abolished LPS induced barrier dysfunction in wild-type mice whereas the same protocol in FerDR mice could only partly inhibit the LPS induced dysfunction. A further reduction in LPS induced dysfunction was not observed on chronic antineutrophil antibody treatment to deplete the resident neutrophil population (data not shown), suggesting that the remaining dysfunction was due to neutrophil independent mechanism(s). Interestingly, neutrophil dependent $^{51}$Cr-EDTA clearance in FerDR mice was approximately double that observed in wild-type mice. As intestinal neutrophil numbers were not significantly different in FerDR mice, compared with wild-type mice, our data would suggest that neutrophils deficient in Fer kinase activity had an enhanced ability to cause damage to the epithelial barrier, perhaps through enhanced release of proteases or reactive oxygen metabolites.
It is unclear at this point which cells are involved in neutrophil independent LPS induced epithelial barrier permeability. One possibility is mast cells, which have previously been shown to be involved in intestinal barrier dysfunction during inflammation associated with ischemia reperfusion,

stress and hypersensitivity,

and parasitic infections.

In addition, we recently demonstrated a role for Fer in mast cell function through regulation of p38 mitogen activated protein kinase,

in response to antigen. However, in that study, mast cell migration and not mediator release was affected by the absence of Fer kinase activity. Another potential site of action might be within the epithelial cell itself. There is a growing body of evidence that suggests a role for Fer in association with adherens junctions and cell-cell contacts,

and therefore a role within tight junction complexes in intestinal epithelial cells is an exciting possibility to be explored.

In summary, we have demonstrated in this study that Fer is involved in regulating neutrophil recruitment (adhesion) to the gut and plays a key role in maintaining intestinal epithelial barrier integrity following LPS challenge. Our data further illustrate that Fer can act through both neutrophil dependent and neutrophil independent mechanisms to modulate intestinal barrier permeability in vivo.

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