The Fps/Fes kinase regulates leucocyte recruitment and extravasation during inflammation

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Summary

Fps/Fes and Fer comprise a distinct subfamily of cytoplasmic protein-tyrosine kinases, and have both been implicated in the regulation of innate immunity. Previous studies showed that Fps/Fes-knockout mice were hypersensitive to systemic lipopolysaccharide (LPS) challenge, and Fer-deficient mice displayed enhanced recruitment of leucocytes in response to localized LPS challenge. We show here for the first time, a role for Fps in the regulation of leucocyte recruitment to areas of inflammation. Using the cremaster muscle intravital microscopy model, we observed increased leucocyte adherence to venules, and increased rates and degrees of transendothelial migration in Fps/Fes-knockout mice relative to wild-type animals subsequent to localized LPS challenge. There was also a decreased vessel wall shear rate in the post-capillary venules of LPS-challenged Fps/Fes-knockout mice, and an increase in neutrophil migration into the peritoneal cavity subsequent to thioglycollate challenge. Using flow cytometry to quantify the expression of surface molecules, we observed prolonged expression of the selectin ligand PSGL-1 on peripheral blood neutrophils from Fps/Fes-knockout mice stimulated *ex vivo* with LPS. These observations provide important insights into the observed *in vivo* behaviour of leucocytes in LPS-challenged Fps/Fes-knockout mice and provide evidence that the Fps/Fes kinase plays an important role in the innate immune response.

Keywords: adhesion molecules; kinase; lipopolysaccharide; neutrophil

Introduction

The *fps/fes* proto-oncogene (hereafter referred to as *fps*), encodes a subgroup IV non-receptor protein-tyrosine kinase.[1,2] The only other known member of this subgroup of kinases is the ubiquitously expressed Fer protein.[2–4] Expression of *fps* has been described in haematopoietic cells including monocytes/macrophages, neutrophils, mast cells, platelets and erythrocytes; as well as in some neuronal, epithelial and vascular endothelial cells. In contrast, during development, *fps* is expressed in all three germ layers[5–7] (reviewed in ref. 8).

The Fps and Fer kinases consist of a C-terminal kinase domain, a central SH_2 domain and an N-terminal Fps/Fer/CIP4 homology (FCH) domain associated with three coiled-coiled domains.[8] This unique N-terminal structure distinguishes Fps and Fer from all other members of the protein-tyrosine kinase family, and it is thought to mediate associations with phospholipid components of membranes as well as the cytoskeleton.[9–11]

The body responds to invading pathogens by initiating an inflammatory response, and this requires the activation of key cell types, including macrophages and neutrophils, which make up the bulk of the body's inflammatory cells.[12] Of great importance in this response is the movement of leucocytes out of the blood, and into surrounding tissues towards the site of injury or infection. The leucocyte does this through an ordered series of events involving interactions between the inflammatory cells and the endothelium. The first step in leucocyte recruitment involves the interaction between selectins on the activated endothelium, and their ligands on the leucocyte, which

mediates leucocyte rolling along the endothelium. The two endothelial selectins primarily responsible for this initial rolling are E-selectin and P-selectin. Evidence for this is demonstrated by the near total lack of leucocyte rolling in mice engineered to harbour a double knockout for these two selectins.[13,14] Furthermore, there is an observed defect in leucocyte rolling in P-selectin single knockout mice, and a defect in slow rolling in E-selectin knockouts.[15] The primary leucocyte ligand for P-selectin,[16–18] and to a lesser extent E-selectin,[19] is CD162, or P-selectin glycoprotein ligand 1 (PSGL-1). Consistent with its role in binding endothelial P-selectin, mice-deficient for PSGL-1 show reduced leucocyte rolling upon tumour necrosis factor-α (TNF-α)-induced inflammation in the cremaster muscle, and a reduced thioglycollate-induced neutrophil influx into the peritoneal cavity; and the magnitudes of these defects are similar to those observed in P-selectin knockout mice.[20] Subsequent to rolling, leucocytes must next achieve firm adhesion along the inflamed vessel before transmigration can take place. One of the proteins involved in this process is the integrin α_M/β_2, also known as Mac-1 or CD11b/CD18. A counter ligand for CD11b/CD18 on the endothelium is intercellular adhesion molecule 1 (ICAM-1). CD11b/CD18 has been reported to participate in both adherence and transmigration of leucocytes.[21]

As a result of the expression of Fps and Fer in macrophages, neutrophils and the vascular endothelium, we have explored the possibility of a role for these tyrosine kinases in the regulation of the innate immune response. We have previously reported increased mortality in Fps knockout ($fps^{-/-}$) mice in response to lipopolysaccharide (LPS) challenge *in vivo*,[22] which was at least partially the result of increased *in vivo* levels of TNF-α caused by enhanced nuclear factor-κB signalling in macrophages.[23] Mice that were deficient in the Fer kinase also displayed increased LPS-induced neutrophil adhesion to venules and extravasation in the cremaster muscle[24] and the small intestine submucosa.[25]

Here we show for the first time that $fps^{-/-}$ mice display increased inflammation as measured by neutrophil rolling, adhesion and extravasation in cremaster venules subsequent to LPS challenge, with concomitant defects in the haemodynamic parameters of these same vessels. Using reflected-light oblique transillumination (RLOT) intravital microscopy, we further show that the rate at which leucocytes cross the cremasteric–endothelial barrier is increased in $fps^{-/-}$ mice. There was also an increase in the number of neutrophils recruited to the peritoneal cavity of $fps^{-/-}$ mice subsequent to thioglycollate challenge. We also observed a prolonged retention of surface PSGL-1 on neutrophils from $fps^{-/-}$ mice subsequent to stimulation with LPS. This latter observation is mechanistically consistent with the observed increases in leucocyte rolling, adhesion and recruitment observed in inflamed tissues of $fps^{-/-}$ mice. Collectively, these observations provide novel evidence implicating Fps in the regulation of innate immunity and the vascular response to inflammation.

Materials and methods

Cremaster surgical preparation and intravital microscopy

Intravital microscopy was performed essentially as described by McCafferty *et al.*[24] with a few exceptions. Briefly, mice were injected subcutaneously with LPS (0·05 μg/kg) into the left side of the scrotum. A 65 mg/kg intraperitoneal injection of Somnotol was used as anaesthetic, 4–0 surgical silk was used for suturing the cremaster muscle, and the suffusate passed over the externalized cremaster muscle was a buffered modified Krebs solution. The LPS used was from *Escherichia coli* serotype 055:B5 (Sigma, Oakville, Canada). After 3 hr, mice were anaesthetized and the contra-lateral cremaster muscle was externalized for intravital video-microscopy recording. Leucocyte rolling, adherence and emigration, were quantified in venules of approximately 30-μm diameter. Rolling flux was calculated as the number of cells rolling past a designated point in the vessel per minute. For assessment of wall shear rate, red blood cell (RBC) velocity was divided by the vessel diameter, and multiplied by 8000. For experiments measuring transmigration rates, we used RLOT microscopy, which makes use of the optical interference phenomena generated by oblique transillumination in conjunction with intravital microscopy. This method utilizes subtle gradients of refractive indices within the tissues for enhanced image contrast as designed by Mempel *et al.*[26] Briefly, the microscope was retrofitted with a 700 ± 20-nm band pass filter in the light path of a 100-W halogen lamp, which illuminated the specimen via a 50/50 beam splitter. Beneath the tissue was placed a 24-mm^2 glass cover slip, painted with aluminium to create a mirror, oriented at an angle of approximately 10° or 15° relative to the stage for the 20 × and 40 × objectives, respectively. Extravasating leucocytes were visualized by RLOT video microscopy in cremaster muscle post-capillary venules of approximately 30-μm diameter. Transmigration rates were calculated as the time elapsed from firm adhesion to tissue migration.

Flow cytometric analysis of adhesion molecule expression on peripheral blood leucocytes

Age-matched mice were killed by inhalation of chloroform. Chest cavities were opened, and blood was removed by cardiac puncture with a 1 ml syringe fitted with a 26-gauge needle using 0·3% tri-sodium citrate as anticoagulant. Blood was then stimulated with LPS at

the indicated times and concentrations. Samples were washed twice with, and resuspended in 300 μl cold PAB [phosphate-buffered saline with 0·3% (wt/v) bovine serum albumin, 0·1% (wt/v) sodium azide]. Samples were incubated with 1·5 μg/ml fluorescein isothiocyanate-conjugated CD11b antibody or 1·0 μg/ml phycoerythrin-conjugated PSGL-1 (CD162) antibody (BD Pharmingen, Mississauga, ON), for 15 min on ice and washed twice with PAB. Samples were then incubated for 3 min at 4° in ACK buffer [154 mM ammonium chloride, 10 mM potassium bicarbonate, 100 μM ethylenediaminetetraacetic acid (EDTA)] to lyse red blood cells. Finally, cells were washed and resuspended in 500 μl PAB, vortexed, combined with 500 ml paraformaldehyde–zinc fixative (Electron Microscopy Sciences, Fort Washington, PA), and analysed by flow cytometry. Neutrophils were gated using forward and side scatter; monocytes/lymphocytes were gated using forward and side scatter, and monocytes were further separated on the basis of CD11b expression.

Leucocyte recruitment assay

Mice were injected intraperitoneally with 1 ml 4% thioglycollate in phosphate-buffered saline, using a 1-ml syringe fitted with a 26-gauge needle. Four hours after injection, peritoneal lavage was performed twice per mouse with 5 ml prewarmed lavage media [RPMI-1640 with 10 mM HEPES, 5 mM EDTA, 10 U/ml heparin, 1% (v/v) antibiotic–antimycotic (Invitrogen, Burlington, Canada), 50 μM α-monothioglycerol]. Following lavage, cells were washed, counted on a Beckman Coulter Z1 particle counter and resuspended at 1×10^6 cells/ml in PAB. Next, 200 μl was transferred into 4-ml snap-cap tubes with a final concentration of 0·2 μg/ml phycoerythrin-conjugated Ly6G antibody (eBioscience, San Diego, CA) and 1·5 μg/ml fluorescein isothiocyanate-conjugated CD11b antibody, as indicated, for 15 min on ice. Cells were then washed and resuspended in 500 μl PAB, vortexed, added to 500 μl formaldehyde–zinc fixative, and analysed by flow cytometry as indicated.

Mice

All animals used in this study were inbred SvJ/129 mice, between 7 and 12 weeks old. The *fps*$^{-/-}$ strain of mice has been described previously.[22] All experiments were carried out according to the guidelines of the Canadian Council on Animal Care, with the approval of the institutional animal care committee.

Statistics

All error bars represent SEM. For Figs 1, 2 and 5, and Table 1, statistical values were calculated by two-way repeated measures analysis of variance (ANOVA) using GRAPH PAD PRISM 4 software. All other reported statistical values were calculated using the Student's *t*-test. All statistical values reported represent a comparison of wild-type versus *fps*$^{-/-}$.

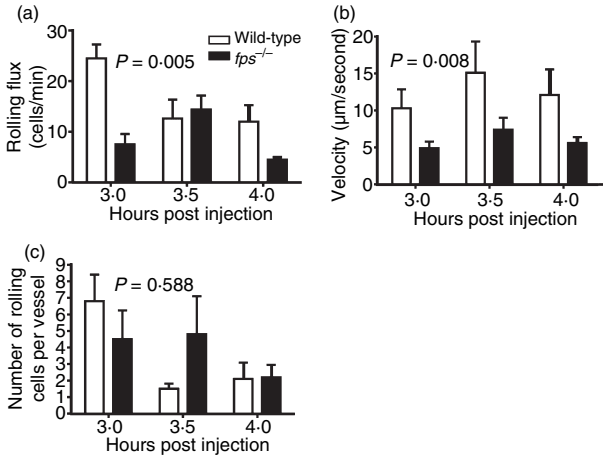

Figure 1. Defects in leucocyte rolling in the cremaster venules of *fps*$^{-/-}$ mice challenged with LPS. Mice were injected intrascrotally with 0·05 μg/kg LPS. The contralateral cremaster muscle was externalized for observation, and the indicated parameters were assessed by intra-vital video microscopy. Number of rolling cells per vessel was calculated by dividing rolling flux by rolling velocity, and multiplying by vessel length (100 μm). *P* values were obtained by two-way ANOVA for repeated measures, and are a comparison of wild type vs. *fps*$^{-/-}$. For all time points, $n = 4$ or 5.

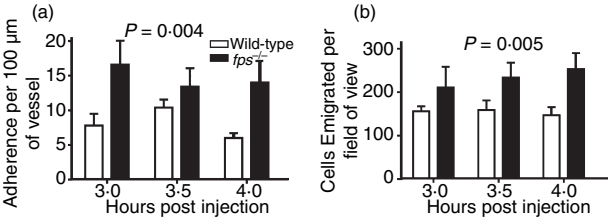

Figure 2. Enhanced leucocyte adherence and emigration in the cremaster venules of *fps*$^{-/-}$ mice challenged with LPS. Mice were injected introscrotally with 0·05 μg/kg LPS. The contralateral cremaster muscle was externalized for observation, and the indicated parameters were assessed by intra-vital video microscopy. *P* values were obtained by two-way ANOVA for repeated measures, and are a comparison of wild type vs. *fps*$^{-/-}$. For all time points, $n = 4$ or 5.

Results

Fps regulates the local inflammatory response to challenge with LPS

Upon localized LPS challenge, leucocytes and the endothelium each follow an ordered sequence of progressive activation events, ultimately resulting in emigration of activated leucocytes out of the vessels into the surrounding

Table 1. Haemodynamic parameters in post-capillary venules of wild-type and *fps*$^{-/-}$ mice after treatment with LPS

Genotype	Time post LPS treatment	Vessel diameter (μm)	Red blood cell velocity (mm/second)	Wall shear **rate (per second)
Wild-type	3 hr	26·9 ± 2·0	1·08 ± 0·05	250 ± 40
Wild-type	3·5 hr	28·6 ± 2·1	1·48 ± 0·30	255 ± 40
Wild-type	4 hr	28·6 ± 2·1	1·30 ± 0·17	197 ± 11
Fps-null	3 hr	30·0 ± 2·8	0·98 ± 0·07	164 ± 4
Fps-null	3·5 hr	28·3 ± 2·9	1·08 ± 0·10	177 ± 8
Fps-null	4 hr	28·8 ± 2·7	1·06 ± 0·04	200 ± 4

Mice were injected intrascrotally with 0·05 μg/kg LPS. The contralateral cremaster muscle was externalized for observation, and the indicated parameters were assessed by intravital video microscopy. Vessel diameter and red blood cell velocity were measured, and shear was calculated as described in the Materials and methods. **$P = 0·021$ for wall shear rate; $P = 0·066$ for red blood cell velocity. *P*-values were obtained by two-way ANOVA for repeated measures, and are a comparison of wild type versus *fps*$^{-/-}$. For all time-points, $n = 5$.

tissue.[27] During this transmigration process, inflammatory cells initially loosely tether to the vessel, allowing them to roll along its surface. Rolling gradually slows, until a firm adhesion and subsequent emigration of the leucocyte across the vessel wall into surrounding tissue is achieved.[27] To explore the potential role of Fps in LPS-induced leucocyte transmigration, we compared the *in vivo* response to localized LPS challenge in wild-type and *fps*$^{-/-}$ mice using a cremaster muscle intravital microscopy model.[24]

Rolling flux is directly related to the number of rolling leucocytes, but inversely related to their rates of rolling, which is a function of their degree of activation. Rolling flux thus serves as a useful indicator of the state of the inflammatory response, with lower values corresponding to higher degrees of inflammation. Leucocytes in *fps*$^{-/-}$ mice had a lower rolling flux at 3 and 4 hr post injection compared to those in wild-type mice, and a two-way ANOVA analysis across the 3-, 3·5- and 4-hr time-points indicated a statistically significant reduction in rolling flux in *fps*$^{-/-}$ mice (Fig. 1a; $P = 0·001$). Leucocyte rolling velocities were significantly higher in wild-type mice than *fps*$^{-/-}$ mice at 3, 3·5, and 4 hr post LPS injection (Fig. 1b; $P = 0·008$). The number of cells rolling along a 100-μm section of vessel was calculated by dividing the rolling flux by the rolling velocity. At 3 hr post LPS challenge, wild-type and *fps*$^{-/-}$ vessels contained the same number of rolling cells. However, by 3·5 hr post challenge, the number of rolling cells in wild-type vessels had decreased dramatically, suggesting that the inflammation was beginning to resolve. In contrast, a reduction in rolling leucocytes was not observed in *fps*$^{-/-}$ vessels until 4 hr post LPS injection (Fig. 1c), indicating a more prolonged inflammatory state in the *fps*$^{-/-}$ mice.

The number of fully adherent leucocytes per 100 μm of vessel was also greater in *fps*$^{-/-}$ mice at all time-points examined, which again suggested that these mice were in a hyper-inflammatory state as compared to wild-type animals (Fig. 2a; $P = 0·004$). Consistent with these observed decreases in leucocyte rolling velocity and increases in numbers of adherent leucocytes, increased numbers of leucocytes had emigrated into the surrounding tissues of *fps*$^{-/-}$ mice at all time-points examined (Fig. 2b; $P = 0·005$).

We also examined haemodynamic parameters in the cremaster muscle venules of LPS-challenged mice.[28] There were no differences in red blood cell velocity between genotypes. However, a lower vessel wall shear rate was observed in LPS-challenged *fps*$^{-/-}$ mice (Table 1; $P = 0·021$), which was consistent with the increased number of adherent cells observed in *fps*$^{-/-}$ mice.

Leucocytes from *fps*$^{-/-}$ mice displayed increased rates of transmigration in response to a local LPS challenge

Having observed an increase in both the number of leucocytes adhered to the vessel wall, and the number of extravasated leucocytes in *fps*$^{-/-}$ mice, we next sought to determine if there might be a difference in the rate at which leucocytes were undergoing diapedesis in wild-type and *fps*$^{-/-}$ mice. RLOT analysis revealed that leucocytes transmigrated the vessels in *fps*$^{-/-}$ mice over 30% faster than in wild-type animals (Fig. 3, 8·0 ± 1·1 min versus 11·8 ± 0·7 min; $P = 0·032$). Together with the parameters examined above, this confirmed that mice lacking Fps were more sensitive to inflammation induced by the localized injection of LPS.

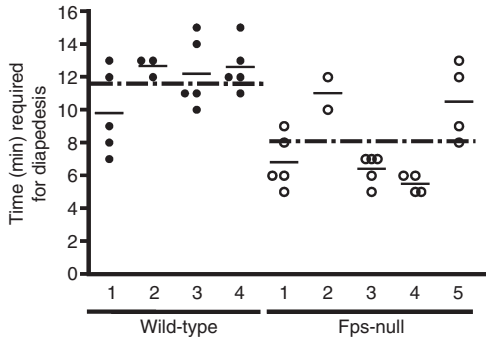

Figure 3. Fps regulates time required for leucocyte diapedesis in response to local challenge with LPS. RLOT was used to assess transmigration of leucocytes by intra-vital video microscopy. Diapedesis time was recorded as the time required for individual leucocytes to escape from vessel into surrounding tissue after initial adherence. Data for each mouse is shown in separate columns. Mean for each mouse is represented by a solid line, and mean for each genotype is represented by a broken line. Student's *t*-test $P < 0·0001$, wild type vs. *fps*$^{-/-}$.

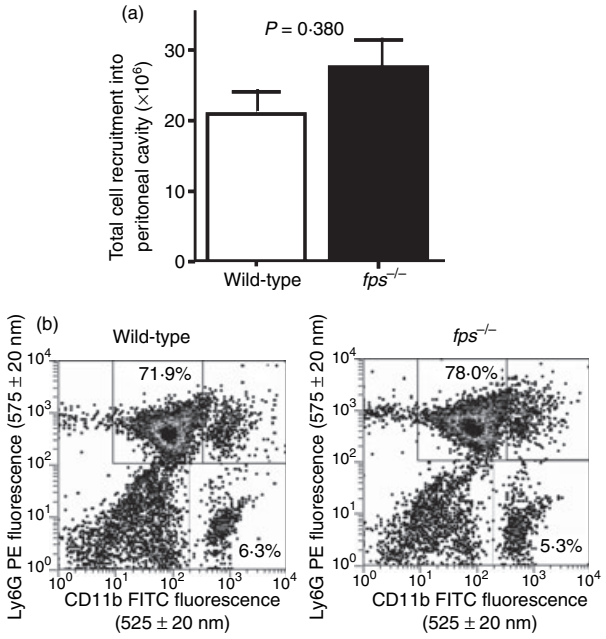

Figure 4. Effect of Fps on neutrophil migration into the peritoneal cavity in response to thioglycollate. Mice were challenged I.P. with 1 ml of 4% thioglycollate, and 4 hr later were euthanized and peritoneal lavage performed. (a) Total cell number recovered by lavage. *P* value was obtained by Student's *t*-test. $n = 5$. (b) Assessment of Ly6G and CD11b expression of recruited cells as measured by flow cytometry. $n = 3$. Ly6G/CD11b+ve $P = 0{\cdot}025$; Ly6G−ve/CD11b+ve $P = 0{\cdot}574$ by Student's *t*-test.

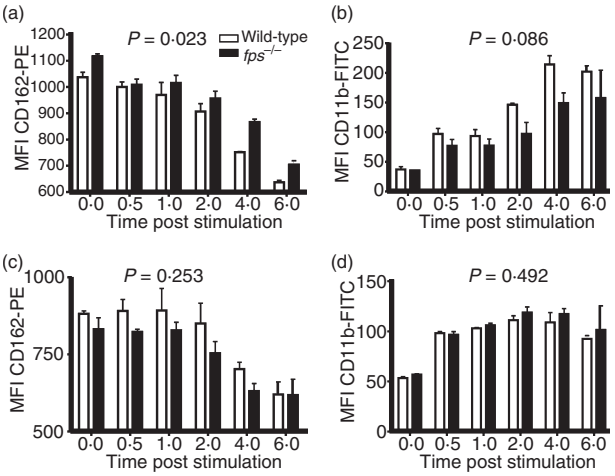

Figure 5. Effect of Fps on PSGL-1 and CD11b expression on peripheral blood neutrophils stimulated with LPS. Peripheral blood was collected by cardiac puncture, and stimulated with LPS at 100 ng/ml. At the indicated times, an aliquot of blood was removed and placed in TBS-V on ice. Expression of PSGL-1 and CD11b was then measured by flow cytometry. (a, b) Neutrophils; (c, d) monocytes. (a, c) Levels of surface expression of PSGL-1. (b, d) Levels of surface expression of CD11b. For all time points, $n = 3$. For all plots, *P* values were obtained by two-way ANOVA for repeated measures, and are a comparison of wild type vs. *fps*$^{-/-}$.

Fps regulates neutrophil migration into the peritoneal cavity in response to thioglycollate

Having observed an increase in the number of extravasated leucocytes in the tissues surrounding the cremaster muscle in *fps*$^{-/-}$ mice, we next asked if this result could be replicated in the peritoneal cavity. To address this, mice were injected intraperitoneally with thioglycollate to induce a peritoneal inflammation, and we then examined the number and composition of cells recovered by peritoneal lavage. As expected, there was an increase in the total number of cells recruited to the peritoneal cavities of *fps*$^{-/-}$ mice (Fig. 4a). Flow cytometric analysis of these peritoneal cells using lineage-specific surface markers revealed a significant increase in the percentage of Ly6G/CD11b double-positive neutrophils recovered from the peritoneum of *fps*$^{-/-}$ mice (Fig. 4b; $P = 0{\cdot}025$). We also determined the percentage of Ly6G^{-} CD11b^{+} cells recruited to the peritoneal cavity, but no difference was found between wild-type and fps$^{-/-}$ mice (Fig. 4b).

To address the possibility that differences in apoptosis might play a role in the observed increases in *fps*$^{-/-}$ elicited neutrophils, we assessed the percentages of annexin V staining Ly6G^{+} cells in the peritoneal lavages of mice at 4 and 24 hr after thioglycollate challenge. While there was a trend toward reduced apoptosis in the *fps*$^{-/-}$ neutrophils at 24 hr, this did not reach statistical significance (24·9 ± 1·7 wild-type versus 18·5 ± 0·1 *fps*$^{-/-}$; $P = 0{\cdot}061$).

Fps regulates LPS-induced changes in PSGL-1 and CD11b surface expression on peripheral blood neutrophils

It is thought that PSGL-1 is one of the major leucocyte adhesion molecules responsible for the initiation of rolling along the activated endothelium, through its binding to P-selectin, and to a lesser extent E-selectin.[19] The integrin CD11b is known to participate in inflammation-induced leucocyte adherence to vessel endothelium. Since we observed aberrant behaviour of *fps*$^{-/-}$ leucocytes with respect to both rolling and adherence, we examined the expression of both of these molecules on peripheral blood neutrophils from wild-type and *fps*$^{-/-}$ mice stimulated *ex vivo* with LPS. In peripheral blood neutrophils from *fps*$^{-/-}$ mice, there was an increased retention of PSGL-1 surface expression after LPS stimulation, as compared to those isolated from wild-type mice (Fig. 5a; $P = 0{\cdot}023$). In the same experiment we also assessed the levels of CD11b surface expression subsequent to LPS stimulation. Surprisingly, we observed a trend toward reduced LPS-induced CD11b up-regulation on *fps*$^{-/-}$ neutrophils, although this difference did not reach statistical significance (Fig. 5b; $P = 0{\cdot}086$). An analysis of PSGL-1 and CD11b expression subsequent to LPS stimulation on monocytes was also performed. As with neutrophils,

monocytes showed a decrease in the level of PSGL-1 surface expression, with a corresponding increase in CD11b levels; however, no difference between wild-type and *fps*$^{-/-}$ cells was observed (Fig. 5c,d).

Discussion

Using mice targeted with a loss-of-function mutation in *fer*, previous studies have established a role for Fps-related Fer kinase in the physiological response of leucocytes to challenge with LPS.[24] We have also previously implicated Fps in the regulation of inflammation by showing that *fps*$^{-/-}$ mice are more susceptible to systemic challenge with LPS[22] and that this defect is probably the result of an overproduction of TNF-α by *fps*$^{-/-}$ macrophages.[23] Here we provide further *in vivo* evidence for the role of Fps in regulating the physiological response to LPS.

In the innate immune response to LPS there is an ordered series of leucocyte–endothelial cell interactions that allow leucocytes to exit the vessel lumen and migrate towards sites of injury or inflammation. This process is one of the hallmarks of inflammation, and is thought to be responsible for much of the resulting tissue damage. Here we show that during every step of this process (leucocyte rolling, adherence, transmigration time and number of extravasated cells), there is a defect in *fps*$^{-/-}$ mice relative to their wild-type counterparts which is consistent with increased inflammation (Figs 1 and 2). In Fig. 1(c), we observed that there was a significant decrease in the number of rolling cells in wild-type vessels between 3 and 3·5 hr post challenge, while in *fps*$^{-/-}$ vessels this decrease was not apparent until 4 hr post challenge, which may indicate a defect or a delay in the resolution of inflammation in *fps*$^{-/-}$ animals. Overall, these defects correspond to a situation in which mice lacking the Fps kinase experience a heightened state of inflammation when challenged locally with LPS. This increase in inflammation in the *fps*$^{-/-}$ mice is most likely detrimental to the animal, which is consistent with the higher mortality rates observed in *fps*$^{-/-}$ mice challenged systemically with LPS, compared to their wild-type cohorts.[22]

The increase in thioglycollate-induced recruitment of neutrophils to the peritoneal cavity of *fps*$^{-/-}$ mice (Fig. 4) is in agreement with the increased leucocyte recruitment to the inflamed cremaster muscle (Fig. 2b). A simple explanation for this difference might be if there are increased numbers of neutrophils in *fps*$^{-/-}$ mice. However, direct analysis of peripheral blood neutrophil levels in unchallenged mice revealed no difference between wild-type and *fps*$^{-/-}$ mice[22] (S.A.P. and P.A.G., unpublished results). With this in mind, the decrease in LPS-induced up-regulation of CD11b in *fps*$^{-/-}$ neutrophils is particularly interesting because previous studies have shown that mice that are deficient for CD11b display increased thioglycollate-induced recruitment of neutrophils into the peritoneal cavity, and that this is the result of a defect in apoptosis in the CD11b-deficient mice.[21] This led us to initially speculate that the increased recovery of peritoneal neutrophils from thioglycollate-challenged *fps*$^{-/-}$ mice (Fig. 4b) might correlate with a defect in apoptosis because of their decreased ability to up-regulate CD11b (Fig. 5b, and see below). However, experiments examining *in vivo* neutrophil apoptosis subsequent to thioglycollate challenge revealed no difference at 4 hr post challenge; and at 24 hr post challenge, while there was a modest decrease in the percentage of apoptotic *fps*$^{-/-}$ neutrophils relative to wild-type, this difference did not reach statistical significance.

Differences in the levels of surface expression of PSGL-1 were also observed on *fps*$^{-/-}$ neutrophils, subsequent to LPS challenge. PSGL-1 is known to be critically important in mediating the rolling of neutrophils along inflamed endothelium.[20] Therefore, our observation that there was increased retention of PSGL-1 on the surface of *fps*$^{-/-}$ neutrophils subsequent to LPS challenge, might at least partially account for the decreases in leucocyte rolling velocity, and the increased number of rolling leucocytes in *fps*$^{-/-}$ mice *in vivo* (Fig. 1b,c). We have previously shown a role for Fps in the regulation of Toll-like receptor 4 and transferrin receptor endocytosis, as well as bacterial phagocytosis, suggesting a more general function for Fps in cytoskeletal reorganization associated with endocytosis or phagocytosis.[23] The surface distribution of PSGL-1 appears to involve a tyrosine kinase-dependent cytoskeletal reorganization process[29] and surface receptor shedding.[30] Likewise, CD11b has been shown to interact with the cytoskeleton (reviewed in ref. 31), changes in which have been proposed to regulate both the mobility of the integrin within the membrane and its binding activity.[32] Therefore, our observations with respect to PSGL-1 and CD11b lend further support to a more generalized function of Fps in cytoskeletal regulation.[8]

The observed differences in PSGL-1 expression are statistically significant; however, the magnitude of the differences is not overly large, with the total area under the curve for *fps*$^{-/-}$ cells being only 9% greater than for wild-type cells. Therefore, although we have established a role for Fps in the regulation of PSGL-1 surface expression, and this is likely to have an effect on leucocyte migration to sites of inflammation, we must also consider the possibility of a role for Fps in other cell types, and in the regulation of other adhesion molecules (see Discussion below).

CD11b has been shown to be a key modulator of neutrophil adherence.[21] We observed increases in leucocyte adherence in *fps*$^{-/-}$ mice *in vivo*, yet surprisingly, there was less CD11b up-regulation on *fps*$^{-/-}$ neutrophils compared to wild-type after *in vitro* LPS challenge. There are several possible explanations for this discrepancy. First, in the cremaster inflammation model used in this study,

leucocyte rolling on the endothelium is thought to be a necessary precursor to leucocyte adherence, and slower leucocyte rolling velocities promote firm adhesion.[33] Therefore, the observed increase in the number of rolling leucocytes and the decrease in leucocyte rolling velocity in *fps*$^{-/-}$ mice (Fig. 1b,c), might account for the observed increase in leucocyte adherence. Second, as shown in Fig. 5(b), although CD11b surface expression was not up-regulated in *fps*$^{-/-}$ neutrophils to the same extent as it was in wild-type cells, it was still up-regulated by over 300% over control levels by 4 hr post LPS stimulation (Fig. 5b, *fps*$^{-/-}$ control mean fluorescence intensity 49·6, mean fluorescence intensity at 4 hr post stimulation = 152·1). Therefore, the level of up-regulation of CD11b in *fps*$^{-/-}$ neutrophils was probably sufficient to achieve firm adhesion, and the increased number of adherent leucocytes in *fps*$^{-/-}$ animals *in vivo* might be the result of the increased number of rolling cells in these animals. Lastly, to facilitate proper adhesion and extravasation, CD11b must change from a low-affinity status to a high-affinity status.[34] Therefore it is possible that even though there was less CD11b expressed on the surface of *fps*$^{-/-}$ cells, a greater proportion of it might have been in the high-affinity state relative to wild-type cells, thereby preferentially promoting the adhesion of *fps*$^{-/-}$ cells.

In addition to PSGL-1 and CD11b, which were examined here, various other adhesion molecules are expressed on neutrophils and are known to play a role in leucocyte adhesion. For instance, a recent article by Hidalgo *et al.* sought to elucidate the complete spectrum of E-selectin-binding partners expressed on neutrophils. Their results demonstrate roles for PSGL-1 in the initiation of rolling, but also for E-selectin ligand 1 in the stabilization and transition to steady rolling, and for CD44 in the control of leucocyte rolling velocities.[35] Interestingly, the authors go on to show that all three E-selectin ligands have a role in regulating neutrophil recruitment in a thioglycollate-induced peritoneal inflammation model.[35] With this in mind, it would be interesting to investigate the possibility that Fps is playing a role in the regulation of other neutrophil adhesion molecules, in addition to PSGL-1 and CD11b.

Gene-targeted Fer-deficient mice[36] also displayed defects in leucocyte behaviour very similar to those observed in the present study.[24] Since Fps and Fer have very similar structures, and might therefore regulate some of the same processes, it is intriguing to speculate that a double *fps–fer* knockout[37] will display an even more severe phenotype than either of the single knockouts. These studies are currently underway.

In addition to leucocyte activation, activation of the vascular endothelium is an essential step in promoting leucocyte rolling, adhesion and extravasation. Fps and Fer are both expressed in vascular endothelial cells as well as in leucocytes; therefore, it will be important to determine how much of the observed hyperinflammatory phenotype seen in either Fps- or Fer-deficient mice is the result of loss of Fps or Fer expression in the endothelium versus in the leucocytes. In support of this idea, a recent report shows a role for cortactin phosphorylation in modulating E-selectin and ICAM-1 clustering and actin remodelling in endothelial cells,[23] thereby affecting PMN transmigration *in vitro*.[38] In that study, Src family kinases were implicated in cortactin phosphorylation. However, other studies have implicated Fer in phosphorylation of cortactin.[36,39,40] Fer activity was also shown to be required for cortactin phosphorylation and strengthening of N-cadherin based cell–cell interactions.[41] Furthermore, preliminary results suggest that Fps might also participate in the phosphorylation of HS-1 (S.A.P. and P.A.G., unpublished observations), which is a cortactin paralogue expressed in haematopoietic cells. Therefore, the possibility exists that Fps may have a role in regulating leucocyte recruitment through its activity in endothelial cells, as well as in leucocytes.

Finally, there is one study implicating Fer in the regulation of another important endothelial adhesion molecule, platelet/endothelial cell adhesion molecule 1 (PECAM-1);[42] and interestingly, PECAM-1 knockout mice have also been shown to have a hyperinflammatory response to LPS. Additional studies will be required to further elucidate the potential roles of Fps and Fer kinases in regulating the cell–cell and cell–matrix receptor systems which control the transendothelial migration of immune cells and cancer cells.

In summary, we show that mice lacking Fps have an increased response to LPS-induced inflammation, with defects in leucocyte rolling, adhesion and extravasation. We go on to show that these defects might be the result of a role for Fps in regulating the surface expression of the adhesion molecule PSGL-1 on the surface of neutrophils. Further investigation into the role of Fps in regulating receptor expression on the surface of endothelial cells is required to elucidate the precise molecular role of this cytoplasmic tyrosine kinase in the inflammatory response.

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References

1 Feldman RA, Gabrilove JL, Tam JP, Moore MA, Hanafusa H. Specific expression of the human cellular fps/fes-encoded protein

NCP92 in normal and leukemic myeloid cells. *Proc Natl Acad Sci USA* 1985; **82**:2379–83.
2 Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. *Oncogene* 2000; **19**:5548–57.
3 MacDonald I, Levy J, Pawson T. Expression of the mammalian c-fes protein in hematopoietic cells and identification of a distinct fes-related protein. *Mol Cell Biol* 1985; **5**:2543–51.
4 Letwin K, Yee SP, Pawson T. Novel protein-tyrosine kinase cDNAs related to fps/fes and eph cloned using anti-phosphotyrosine antibody. *Oncogene* 1988; **3**:621–7.
5 Haigh J, McVeigh J, Greer P. The fps/fes tyrosine kinase is expressed in myeloid, vascular endothelial, epithelial, and neuronal cells and is localized in the trans-golgi network. *Cell Growth Differ* 1996; **7**:931–44.
6 Care A, Mattia G, Montesoro E, Parolini I, Russo G, Colombo MP, Peschle C. c-fes expression in ontogenetic development and hematopoietic differentiation. *Oncogene* 1994; **9**:739–47.
7 Sangrar W, Gao Y, Bates B, Zirngibl R, Greer PA. Activated Fps/Fes tyrosine kinase regulates erythroid differentiation and survival. *Exp Hematol* 2004; **32**:935–45.
8 Greer P. Closing in on the biological functions of fps/fes and fer. *Nat Rev Mol Cell Biol* 2002; **3**:278–89.
9 Tsujita K, Suetsugu S, Sasaki N, Furutani M, Oikawa T, Takenawa T. Coordination between the actin cytoskeleton and membrane deformation by a novel membrane tubulation domain of PCH proteins is involved in endocytosis. *J Cell Biol* 2006; **172**:269–79.
10 Takahashi S, Inatome R, Hotta A, Qin Q, Hackenmiller R, Simon MC, Yamamura H, Yanagi S. Role for Fes/Fps tyrosine kinase in microtubule nucleation through is Fes/CIP4 homology domain. *J Biol Chem* 2003; **278**:49129–33.
11 Laurent CE, Delfino FJ, Cheng HY, Smithgall TE. The human c-Fes tyrosine kinase binds tubulin and microtubules through separate domains and promotes microtubule assembly. *Mol Cell Biol* 2004; **24**:9351–8.
12 Bellingan G. Inflammatory cell activation in sepsis. *Br Med Bull* 1999; **55**:12–29.
13 Frenette PS, Mayadas TN, Rayburn H, Hynes RO, Wagner DD. Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. *Cell* 1996; **84**:563–74.
14 Bullard DC, Kunkel EJ, Kubo H *et al.* Infectious susceptibility and severe deficiency of leukocyte rolling and recruitment in E-selectin and P-selectin double mutant mice. *J Exp Med* 1996; **183**:2329–36.
15 Kunkel EJ, Ley K. Distinct phenotype of E-selectin-deficient mice. E-selectin is required for slow leukocyte rolling *in vivo*. *Circ Res* 1996; **79**:1196–204.
16 Moore KL, Stults NL, Diaz S, Smith DF, Cummings RD, Varki A, McEver RP. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J Cell Biol* 1992; **118**:445–56.
17 Sako D, Chang XJ, Barone KM *et al.* Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell* 1993; **75**:1179–86.
18 Ma L, Raycroft L, Asa D, Anderson DC, Geng JG. A sialoglycoprotein from human leukocytes functions as a ligand for P-selectin. *J Biol Chem* 1994; **269**:27739–46.
19 Asa D, Raycroft L, Ma L, Aeed PA, Kaytes PS, Elhammer AP, Geng JG. The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. *J Biol Chem* 1995; **270**:11662–70.
20 Yang J, Hirata T, Croce K *et al.* Targeted gene disruption demonstrates that P-selectin glycoprotein ligand 1 (PSGL-1) is required for P-selectin-mediated but not E-selectin-mediated neutrophil rolling and migration. *J Exp Med* 1999; **190**:1769–82.
21 Coxon A, Rieu P, Barkalow FJ, Askari S, Sharpe AH, von Andrian UH, Arnaout MA, Mayadas TN. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity* 1996; **5**:653–66.
22 Zirngibl RA, Senis Y, Greer PA. Enhanced endotoxin sensitivity in fps/fes-null mice with minimal defects in hematopoietic homeostasis. *Mol Cell Biol* 2002; **22**:2472–86.
23 Parsons SA, Greer PA. The Fps/Fes kinase regulates the inflammatory response to endotoxin through down-regulation of TLR4, NF-κB activation, and TNF-α secretion in macrophages. *J Leukoc Biol* 2006; **80**:1522–8.
24 McCafferty DM, Craig AW, Senis YA, Greer PA. Absence of Fer protein-tyrosine kinase exacerbates leukocyte recruitment in response to endotoxin. *J Immunol* 2002; **168**:4930–5.
25 Qi W, Ebbert KV, Craig AW, Greer PA, McCafferty DM. Absence of Fer protein tyrosine kinase exacerbates endotoxin induced intestinal epithelial barrier dysfunction *in vivo*. *Gut* 2005; **54**:1091–7.
26 Mempel TR, Moser C, Hutter J, Kuebler WM, Krombach F. Visualization of leukocyte transendothelial and interstitial migration using reflected light oblique transillumination in intravital video microscopy. *J Vasc Res* 2003; **40**:435–41.
27 Inflammation: The Leukocyte Adhesion Cascade: http://bme.virginia.edu/ley/.
28 House SD, Lipowsky HH. Leukocyte-endothelium adhesion: microhemodynamics in mesentery of the cat. *Microvasc Res* 1987; **34**:363–79.
29 Ba X, Chen C, Gao Y, Zeng X. Signaling function of PSGL-1 in neutrophil: tyrosine-phosphorylation-dependent and c-Abl-involved alteration in the F-actin-based cytoskeleton. *J Cell Biochem* 2005; **94**:365–73.
30 Davenpeck KL, Brummet ME, Hudson SA, Mayer RJ, Bochner BS. Activation of human leukocytes reduces surface P-selectin glycoprotein ligand-1 (PSGL-1, CD162) and adhesion to P-selectin *in vitro*. *J Immunol* 2000; **165**:2764–72.
31 Pavalko FM, Otey CA. Role of adhesion molecule cytoplasmic domains in mediating interactions with the cytoskeleton. *Proc Soc Exp Biol Medical* 1994; **205**:282–93.
32 Anderson SI, Hotchin NA, Nash GB. Role of the cytoskeleton in rapid activation of CD11b/CD18 function and its subsequent downregulation in neutrophils. *J Cell Sci* 2000; **113**:2737–45.
33 Jung U, Norman KE, Scharffetter-Kochanek K, Beaudet AL, Ley K. Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment *in vivo*. *J Clin Invest* 1998; **102**:1526–33.
34 Fagerholm S, Varis M, Stefanidakis M, Hilden TJ, Gahmberg CG. α-chain phosphorylation of the human leukocyte CD11b/CD18 (Mac-1) integrin is pivotal for integrin activation to bind ICAMs and leukocyte extravasation in vivo. *Blood* 2006; **108**:3379–86.
35 Hidalgo A, Peired AJ, Wild MK, Vestweber D, Frenette PS. Complete identification of E-selectin ligands on neutro-

phils reveals distinct functions of PSGL-1, ESL-1, and CD44. *Immunity* 2007; **26**:477–89.

36 Craig AW, Zirngibl R, Williams K, Cole LA, Greer PA. Mice devoid of fer protein-tyrosine kinase activity are viable and fertile but display reduced cortactin phosphorylation. *Mol Cell Biol* 2001; **21**:603–13.

37 Senis YA, Craig AW, Greer PA. Fps/Fes and Fer protein-tyrosin-ekinases play redundant roles in regulating hematopoiesis. *Exp Hematol* 2003; **31**:673–81.

38 Yang L, Kowalski JR, Zhan X, Thomas SM, Luscinskas FW. Endothelial cell cortactin phosphorylation by Src contributes to polymorphonuclear leukocyte transmigration *in vitro*. *Circ Res* 2006; **98**:394–402.

39 Fan L, Di Ciano-Oliveira C, Weed SA, Craig AW, Greer PA, Rotstein OD, Kapus A. Actin depolymerization-induced tyrosine phosphorylation of cortactin. the role of Fer kinase. *Biochem J* 2004; **380**:581–91.

40 Kim L, Wong TW. Growth factor-dependent phosphorylation of the actin-binding protein cortactin is mediated by the cytoplasmic tyrosine kinase FER. *J Biol Chem* 1998; **273**:23542–8.

41 El Sayegh TY, Arora PD, Fan L, Laschinger CA, Greer PA, McCulloch CA, Kapus A. Phosphorylation of N-cadherin-associated cortactin by fer kinase regulates N-cadherin mobility and intercellular adhesion strength. *Mol Biol Cell* 2005; **16**:5514–27.

42 Kogata N, Masuda M, Kamioka Y, Yamagishi A, Endo A, Okada M, Mochizuki N. Identification of Fer tyrosine kinase localized on microtubules as a platelet endothelial cell adhesion molecule-1 phosphorylating kinase in vascular endothelial cells. *Mol Biol Cell* 2003; **14**:3553–64.