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Absence of Fer Protein-Tyrosine Kinase Exacerbates Leukocyte Recruitment in Response to Endotoxin

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The group IV cytoplasmic protein-tyrosine kinase Fer has been linked to cellular signaling responses to many different stimuli, including growth factors and cytokines. However, the biological relevance of Fer activation in vivo has not been demonstrated to date. Recently, we generated a transgenic mouse line in which Fer protein is expressed but lacks catalytic activity. Homozygous mutant mice were viable and fertile, and showed no overt defects. In this study, we used intravital microscopy to examine the role of Fer kinase in leukocyte recruitment (rolling adhesion and emigration) in response to LPS challenge in skeletal muscle microcirculation. In addition, we measured vascular permeability changes (FITC-albumin leakage, venular-to-interstitial space) in response to Ag to examine general endothelial cell function. Local administration of LPS induced decreased leukocyte rolling velocity and increased leukocyte adhesion and emigration in wild-type mice. LPS-induced changes in leukocyte rolling velocity and rolling flux were not significantly different in Fer mutants. However, LPS-induced leukocyte adhesion (23 ± 3 vs 11 ± 3 cells/100 μm) and emigration (100 ± 5 vs 28 ± 7 cells/field) were significantly elevated in Fer-mutant mice relative to wild-type mice, respectively, suggesting an essential role for the Fer kinase in regulating inflammation-induced leukocyte emigration. Vascular permeability increases in response to Ag were similar between the two groups, indicating that the ability of endothelial cells to retract is intact in the absence of Fer kinase. These data provide the first evidence for a biological role for Fer in regulation of leukocyte recruitment during the innate immune response.

to study in vivo the leukocyte recruitment cascade (rolling adhesion and emigration) in response to LPS. Leukocyte recruitment from the microcirculation to extravascular space is a hallmark feature of inflammation and is directly associated with tissue damage and dysfunction. We demonstrate that, in the absence of Fer kinase, leukocyte emigration is exaggerated in response to LPS, illustrating for the first time an important physiological role for Fer kinase in regulating innate immunity. In addition, we demonstrate that endothelial function appears normal inasmuch as vascular permeability changes in response to Ag remain intact.

Materials and Methods

Animals

The activity of Fer kinase was inactivated in mice by targeting the fer locus with a kinase-inactivating missense mutation (FerD743R) as previously described (9). These mice were bred at Queen’s University (Kingston, Ontario, Canada), and were used between 6 and 10 wk of age, and weighed between 19 and 28 g. Wild-type controls (129Sv/J) were bred at the same facility and appropriate age-matched controls were used in all experiments. All experimental procedures were approved by the Queen’s University Animal Care Committee and conform to the guidelines established by the Canadian Council for Animal Care.

Intravital microscopy

Mice were anesthetized by i.p. injection with a mixture of 10 mg/kg Xylocaine (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Rogar/STB, Montreal, Quebec, Canada). The left jugular vein was cannulated to administer anesthetic and drugs. An incision was made in the scrotal skin to expose the left cremaster muscle (all local injections were administered on the right side), which was then carefully removed from the associated fascia. A lengthwise incision was made on the ventral surface of the cremaster muscle. The testicle and epididymis were separated from the underlying muscle and placed into the abdomen cavity. The muscle was then spread out over an optically clear viewing pedestal and secured along the edges with 5-0 suture. The exposed tissue was suffused with bicarbonate-buffered saline (pH 7.4, temperature 37°C). The cremasteric microcirculation was observed though an intravital microscope (Wild-Leitz trinocular ELR; Leica Microsystems Canada, Willlowdale, Ontario, Canada) with a ×25 objective lens (Leitz Wetzlar NPL, Hitachi, Tokyo, Japan) and a video recorder (Hitachi SVHS; Hitachi, Tokyo, Japan) as previously described (32, 33). Images of the microcirculation were recorded over a 30- or 60-min time frame and sent to a Matrox Meteor II Multichannel (RGB) Image capture card for online video image processing and analysis with Image-Pro Plus 4.0 software (Media Cybernetics).

A single unbranched cremasteric venule (20–40 μm in diameter) was selected in each mouse for study. Venular diameter (Dv) was measured using Image-Pro Plus 4.0 software. Rolling leukocytes were defined as those leukocytes that rolled at a velocity slower than that of RBCs. Leukocyte rolling velocity was measured for the first 20 leukocytes entering the field of view and was determined as the time required for a leukocyte to traverse a given length of venule. Leukocyte adhesion was quantified as the number of leukocytes that adhered to the vessel wall for 30 s or more within the same area of vessel throughout the experiment. The number of emigrated leukocytes was quantified by counting cells in the extravascular space within the field of view (a region of ~400 x 600 μm) adjacent to the venule under study. RBC velocity (Vv) was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX). Venular blood flow was calculated from the product of cross-sectional area and mean RBC velocity (Vvmean = Vv / Dv), assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: γ = 8 x (Vvmean/Dv) (34).

Local LPS administration

LPS from Escherichia coli 0111:B4 dissolved in pyrogenfree water was added to 0.2 ml sterile saline. This LPS was highly purified, containing <0.01 endotoxin cont. The number of LPS particles was measured locally by s.c. injection to the right cremaster muscle using a 30-gauge needle. This dose of LPS has been shown to be optimal for examination of leukocyte-endothelial interactions in vivo (35, 36). The microcirculation of the cremaster muscle was studied 3.5–4.5 h later by intravital microscopy.

Vascular permeability in response to Ag

To assess general endothelial cell function, in a separate group of mice we measured vascular permeability changes after antigenic challenge in sensitized mice. A rapid retraction of endothelial cells is thought to occur after the release of mediators such as histamine from mast cells and does not require/involve the activation of the circulating leukocytes (37). Mice were systemically (i.p.) sensitized with 10 μg chicken egg OVA mixed with 10 mg grade V aluminum hydroxide (both from Sigma-Aldrich, St. Louis, MO), in a total volume of 0.2 ml saline. Two weeks later, mice were anesthetized and prepared for intravital microscopy as described. A control reading of the leukocyte-endothelial interactions in the cremasteric microvasculature was obtained before challenge with 50 μg/ml OVA in buffer solution. The immediate hypersensitivity response was observed over the next 60 min. The degree of vascular albumin leakage from cremasteric venules was quantified as previously described (38). Briefly, FITC-labeled bovine albumin (25 mg/kg; Sigma-Aldrich) was administered to the mice i.v. at the start of the experiment, and FITC-derived fluorescence (excitation wavelength, 450–490 nm; emission wavelength, 520 nm) was detected using a silicon-enhanced charge-coupled device camera (model C-2400-08; Hamamatsu Photonics, Hamamatsu City, Japan). Image analysis software (Optimas; Bioscan, Washington, DC) was used to determine the intensity of FITC-albumin-derived fluorescence within the lumen of the venule and in the adjacent perivascular tissue. Background was defined as the fluorescence intensity before FITC-albumin administration. The index of vascular albumin leakage was determined according to the following ratio expressed as a percentage: (mean interstitial intensity − background)/ (venular intensity − background) (38).

Passive cutaneous anaphylaxis reaction

Blood was obtained from all OVA-sensitized animals at the end of the experiment by intracardiac puncture to generate serum. Serial dilutions (1/8–1/64) of the serum samples were prepared and 200 μl of each sample was injected intradermally into the shaved backs of untreated Sprague Dawley rats. Seventy-two hours later, the rats were challenged systemically with a solution of 5 mg chicken egg OVA in 1.5 ml saline containing 2.5 mg Evan’s blue dye. Sixty minutes later, the highest dilution that produced a distinct blue region (Evan’s blue dye leakage) at the center of the injection site was read as the Ab titer (39, 40). All animals used had serum anti-OVA Ab titers of at least 1/8.

Circulating leukocyte counts

At the end of each experiment, whole blood was obtained via cardiac puncture and total leukocyte counts were performed using a Bright-line hemocytometer (Hauser Scientific, Horsham, PA). Peripheral blood analysis was also performed on nonchallenged, age- and sex-matched wild-type and Fer-deficient mice (7 mo age; n = 6 per genotype). Mice were deeply anesthetized with chloroform, and peripheral blood (0.7 ml) was collected by cardiac puncture and quickly mixed with 10% w/v EDTA (10 μl). Total white blood cell (WBC) counts and five-part differential counts were measured using a hematology analyzer (Sysmex XE-2100; Roche Diagnostics, Indianapolis, IN).

Statistical analysis

Data are expressed as the mean ± SEM. Groups of data were compared using nonparametric Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance.

Results

Leukocyte kinetics in response to local LPS

LPS treatment in wild-type mice significantly decreased leukocyte rolling velocity (Fig. 1A) from 41.6 ± 6.4 μm/sec to 20.3 ± 4.1 μm/sec, indicating a local activation of the vasculature. A similar LPS-induced decrease in leukocyte rolling velocity was observed in Fer-mutant mice (Fig. 1A) 3.5–4.5 h post-intrascrotal LPS administration. During this same period, leukocyte rolling flux in both LPS-challenged and untreated wild-type mice decreased from ~80 to 40 cells/min (Fig. 1B). Interestingly, over this same observation period leukocyte rolling flux in LPS-challenged Fer-mutant mice remained relatively constant at ~50 cells/min.
A significant increase in leukocyte adhesion was observed in wild-type mice cremasteric microvasculature between 3.5 and 4.5 h post-LPS administration (Fig. 2). In the microvasculature of Fer-mutant mice we observed a greater increase in leukocyte adhesion to an average of 28 cells/100 μm at 4 and 4.5 h post-LPS administration. Although the difference at these two later time points was not statistically significant between wild-type and Fer-mutant mice, there was a significant difference between genotypes at the 3.5-h time point. Increased adhesion in Fer mutants is consistent with the reduced adhesion that was previously reported in Fer-overexpressing cells (29).

Hemodynamic parameters after local LPS

The hemodynamic parameters in intrascrotal LPS-treated wild-type or Fer-mutant mice are shown in Table II. These data illustrate a significant drop in RBC velocity and calculated shear wall rates in Fer-mutant mice 4.5 h post-local LPS administration without a significant change in vessel diameter.

Vascular permeability in response to Ag challenge

To assess general endothelial function in the absence of Fer kinase, vascular permeability changes were observed in response to Ag in sensitized mice. Fig. 4 illustrates the index of vascular albumin

Table 1. Total peripheral WBC counts and differential analysis from untreated wild-type or Fer-mutant mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>Wild Type</th>
<th>Fer-Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC count</td>
<td>5.53 ± 1.20</td>
<td>6.17 ± 0.75</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.11 ± 0.18</td>
<td>1.59 ± 0.19</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>4.17 ± 1.05</td>
<td>4.29 ± 0.82</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.09 ± 0.07</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.10 ± 0.05</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.07 ± 0.05</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
</table>

*Data (× 10^9 cells per liter) are expressed as the mean ± SEM; n = 6.
leakage from the cremaster expressed as a percentage: interstitial/venular (38). Before challenge, the FITC-albumin interstitial:venular ratio equilibrated at similar levels in both wild-type and Fer-mutant mice (10–20%). This level is not different from a standard baseline obtained in nonsensitized or untreated wild-type mice (data not shown). Within 5 min of challenge with OVA in the buffer perfusate a significant increase in vascular permeability could be observed in both wild-type and Fer-mutant mice, which was maintained for the duration of the experiment. No significant differences were observed between the two groups, indicating that the ability of endothelial cells to retract was intact in the absence of Fer kinase. All data illustrated comes from mice with a positive passive cutaneous anaphylaxis reaction.

Leukocyte kinetics in sensitized wild-type and Fer-mutant mice

Under baseline conditions, leukocyte kinetics including rolling flux, adhesion, and emigration did not differ significantly between sensitized wild-type and sensitized Fer-mutant mice, and these values were not significantly different from baseline data from untreated wild-type mice (Table III). After Ag challenge a gradual increase in leukocyte adhesion can be observed over 1 h in wild-type sensitized mice (Fig. 5). We have previously shown that this adhesion results in significant leukocyte recruitment by 4 h post-challenge (33). At 60 min post-Ag challenge, leukocyte recruitment in Fer-mutant mice is not significantly elevated from pre-challenge values (Fig. 5). These data may suggest a delayed effect on leukocyte recruitment at later time points in the absence of Fer kinase.

Hemodynamic parameters

Table IV outlines the hemodynamic parameters (vessel diameter, RBC velocity, and calculated vessel wall shear rate) from skeletal muscle postcapillary venules in untreated wild-type, sensitized wild-type, or sensitized Fer-mutant mice. Under baseline conditions sensitized wild-type and Fer-mutant mice had similar hemodynamic parameters. After antigenic challenge hemodynamic parameters did not significantly differ from baseline values in any group studied. The mean circulating WBC count in untreated wild-type mice was 7.6 ± 0.9 × 10⁹/ml (data not shown). WBC counts in sensitized wild-type mice or Fer-mutant mice did not differ significantly from untreated wild-type mice (data not shown).

Discussion

In this study we have used Fer-mutant mice to examine the physiological relevance of Fer kinase in response to LPS challenge. We have demonstrated, for the first time, that Fer plays an essential role in the leukocyte recruitment cascade in response to endotoxin in vivo. Using intravital microscopy we demonstrated that Fer-mutant mice have a greater ability to recruit leukocytes in response to the same local dose of LPS than Fer kinase-competent mice. This effect appears to be on the ability of cells to adhere and emigrate and not an effect on leukocyte rolling. Such a profound effect on leukocyte emigration would suggest that there is no compensation of Fer activity with the closely related Fps/Fes PTK. Preliminary experiments with double knockout mice where both Fer and Fps/Fes are inactivated (A. Craig, Y. Senis, and P. Greer,
manuscript in preparation) have revealed similar leukocyte recruitment levels to support this (our unpublished data).

In this study we observed that Fer-mutant mice but not wild-type mice respond to LPS with reduced hemodynamic stability (decreased shear in postcapillary venules). This effect alone would not be sufficient to account for the profound emigration observed in Fer-mutant mice, as previous studies have shown that leukocyte recruitment is not dependent on shear forces alone (41). A sequential cascade of leukocyte-endothelial cell interactions mediates the recruitment of leukocytes from the circulation to a site of injury or inflammation (42–44). Leukocyte tethering and rolling are the first interactions that occur between circulating leukocytes and vascular endothelial cells. Rolling leukocytes can then be activated to firmly adhere to the vascular endothelium and subsequently emigrate between endothelial cells into the extravascular space. These interactions are mediated by adhesion molecule expression on endothelial cells and leukocytes. The ubiquitous expression pattern of Fer allows for an effect within the endothelial cells, circulating leukocytes, or extravascular cells such as mast cells or macrophages. It remains to be investigated whether the adhesive mechanisms in multiple cell types are the same in Fer-mutant and wild-type mice in response to LPS.

We also examined the role of Fer in endothelial cell permeability increases in response to Ag. During sensitization, mast cells bind IgE through high-affinity FceRI receptors. Upon subsequent antigenic challenge mast cells are induced to rapidly degranulate, releasing preformed mediators which initiate the allergic response, inducing increased vascular permeability and leukocyte recruitment (45). In 1995, Penhallow et al. (27) demonstrated that in vitro Fer was temporally activated in sensitized PT18 mouse mast cell line challenged with Ag, suggesting a role in the immediate allergic response. However, in this study we failed to demonstrate a role for Fer kinase in vivo in vascular permeability changes during an IgE-mediated hypersensitivity reaction. These data demonstrate that, in the absence of Fer kinase, endothelial cells retain the ability to retrace to increase vascular permeability in the microcirculation. However, mice lacking Fer kinase may have an altered response with respect to subsequent leukocyte recruitment during the late-phase reaction, and this certainly warrants further investigation.

Fer activation may be involved in LPS-activated cellular responses by a direct activation downstream of LPS receptor (CD14/TLR4) or by an indirect activation downstream of cytokine receptors activated by cytokines induced by LPS. Evidence exists for Fps/Fes activation in response to ligand binding to various cytokine receptors (22–24). Because Fps/Fes and Fer share 70% homology in their tyrosine kinase domains and many of the antisera used are not specific enough to distinguish between the two PTKs, the possibility arises that some of these previous studies may have been detecting Fer activation. The difference we observed between LPS-induced and Ag-induced responses may be indicative of differences between new mediator synthesis and preformed/stored mediator release. Therefore, increased cytokine production as a result of LPS receptor signaling or a direct effect on gene regulation cannot be discounted in the Fer mutants.

Recent evidence has demonstrated an association between Fer and cadherin-catenin complexes and cytoskeletal elements. For example, Fer has been shown to be constitutively associated with catenin-related p120 protein through its N-terminal region (25). In addition, Fer was recently found to be associated with N-cadherin and focal adhesion complexes in retinal studies of neurite outgrowth (30, 31). Therefore, Fer has the potential to act as a regulator of 1) leukocyte cell contractile components required for cell mobility/motility, 2) endothelial cell-cell junctions to enable retraction to accommodate emigrating leukocytes, and 3) adhesion molecule function for cell-cell or cell-matrix contact. Our data showing no effect on endothelial permeability in response to Ag argue against the second possibility. However, it should be noted that we have previously shown that Fer activation is not required for p120<sup>th</sup> or β-catenin phosphorylation in response to growth factors (platelet-derived growth factor or epidermal growth factor) (9); therefore, Fer involvement in cadherin-catenin complexes and focal adhesions would appear to be stimulus specific.

It is unknown at this stage which signaling pathways are involved in activating Fer kinase or are acted upon by Fer kinase. Recently we have shown that the ability of leukocytes to emigrate can be blocked by inhibition of p38 mitogen-activated protein kinase (MAPK) (46), demonstrating a dominant role for p38 MAPK pathway in leukocyte recruitment in vivo, and raises the possibility that Fer kinase may regulate this pathway in some manner. Indeed, it is well documented that LPS activates p38 MAPK, warranting investigation of the role of group IV protein kinases in p38 MAPK activation.

Our data demonstrate for the first time that Fer kinase plays an essential role in vivo to regulate/dampen cellular recruitment, especially emigration, in response to endotoxin. This involvement appears to be stimulus specific in as much as vascular permeability increases in response to Ag are normal in the absence of Fer kinase. The latter requires no cytokine production to elicit an inflammatory response. The cellular signaling pathways involved in Fer kinase activation and the mechanism by which Fer regulates leukocyte recruitment in response to endotoxin remain to be elucidated; however, these data target Fer kinase as a key regulator of the innate immune response. Interestingly, innate immune defects have also been observed in mice lacking Fps/Fes (11, 47). Use of compound mutant mice deficient in Fer and Fps/Fes should allow us to test for functional redundancy between these two highly related kinases.

Acknowledgments
We thank Dr. Chris Chapler for the use of his laboratory and we gratefully acknowledge Jeff Mewburn for his assistance with the experiments for the completion of this study.

### Table IV. Hemodynamic parameters in sensitized wild-type or Fer-mutant mice before and 5 min after challenge with Ag OVA<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Buffer-Challenged Untreated Wild Type</th>
<th>OVA-Challenged Sensitized Wild Type</th>
<th>OVA-Challenged Sensitized Fer-Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>Vessel diameter (µm)</td>
<td>30.9 ± 0.3</td>
<td>30.9 ± 0.3</td>
<td>23.5 ± 0.7</td>
</tr>
<tr>
<td>RBC velocity (mm/s)</td>
<td>2.0 ± 0.03</td>
<td>1.9 ± 0.07</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Wall shear rate (s&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>319 ± 7.0</td>
<td>313 ± 12</td>
<td>388 ± 32</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are expressed as the mean ± SEM; n = 3.
References


