Nuclear-localized focal adhesion kinase regulates inflammatory VCAM-1 expression

Vascular cell adhesion molecule–1 (VCAM-1) plays important roles in development and inflammation. Tumor necrosis factor–α (TNF-α) and focal adhesion kinase (FAK) are key regulators of inflammatory and integrin–matrix signaling, respectively. Integrin costimulatory signals modulate inflammatory gene expression, but the important control points between these pathways remain unresolved. We report that pharmacological FAK inhibition prevented TNF-α–induced VCAM-1 expression within heart vessel–associated endothelial cells in vivo, and genetic or pharmacological FAK inhibition blocked VCAM-1 expression during development. FAK signaling facilitated TNF-α–induced, mitogen-activated protein kinase activation, and, surprisingly, FAK inhibition resulted in the loss of the GATA4 transcription factor required for TNF-α–induced VCAM-1 production. FAK inhibition also triggered FAK nuclear localization. In the nucleus, the FAK-FERM (band 4.1, ezrin, radixin, moesin homology) domain bound directly to GATA4 and enhanced its CHIP (C terminus of Hsp70-interacting protein) E3 ligase–dependent polyubiquitination and degradation. These studies reveal new developmental and anti-inflammatory roles for kinase-inhibited FAK in limiting VCAM-1 production via nuclear localization and promotion of GATA4 turnover.

Introduction

Vascular cell adhesion molecule–1 (VCAM-1) is a transmembrane protein of the immunoglobulin superfamily expressed on blood vessels after endothelial cell (EC) stimulation by inflammatory cytokines such as tumor necrosis factor–α (TNF-α; Osborn et al., 1989). VCAM-1 mediates leukocyte binding to the vascular endothelium via α4β1 or α4β7 integrins, where signaling events triggered by VCAM-1 and integrin binding contribute to the development of atherosclerosis and rheumatoid arthritis (Carter and Wicks, 2001; Libby, 2002). VCAM-1 is also expressed on the mouse embryo allantois and binds to α4β1 integrin on the chorion to facilitate chorioallantoic fusion and placental development (Gurtner et al., 1995; Kwee et al., 1995; Inman and Downs, 2007). TNF-α triggers increased VCAM-1 production via several intracellular signaling cascades, including MAPK and nuclear factor–κ light chain enhancer of activated B cells (NF-κB) pathways (Pober, 2002; Karin and Gallagher, 2009). These signaling cascades affect multiple transcription factors, including activating protein complex–1 (AP-1), NF-κB, and GATA-binding proteins that bind to the VCAM-1 promoter (Ahmad et al., 1998; Molkentin, 2000; Minami and Aird, 2001). It is a combination of transcription factor phosphorylation and stability that contributes to TNF-α–induced VCAM-1 promoter activation.

TNF-α–induced signaling is highly context dependent, triggering either cell death (cytotoxic activity) or cell activation (proinflammatory activity; MacEwan, 2002). Costimulatory signals from the ECM play an active role in preventing TNF-α cytotoxic activity and also a permissive function in facilitating TNF-α proinflammatory EC activation (Bieler et al., 2007). Transmembrane integrin receptors bind to ECM proteins and...
facilitate the activation of nonreceptor protein tyrosine kinases (PTKs), MAPKs, and NF-κB pathways (Schwartz, 2001). Intergi
mediated signaling supports TNF-α–induced cell survival and gene expression (Fornaro et al., 2003). This requires NF-κB ac
activation (Beg and Baltimore, 1996) and PTKs such as Syk, and Src family PTKs have been linked to TNF-α–induced NF-κB ac
activation (Huang et al., 2003; Takada and Aggarwal, 2004). However, integrin and FAK PTK activation are more strongly
associated with TNF-α–induced MAPK activation (Short et al., 1998; Schlaepfer et al., 2007; Young et al., 2010). Although
PTK activity is needed for TNF-α–induced VCAM-1 expression (Weber et al., 1995; May et al., 1996), the PTKs facilitat
ing these signaling events remain unknown.

FAK is comprised of an N-terminal FERM (band 4.1, ezrin, radixin, moesin homology) domain, central PTK region,
and a C-terminal (CT) domain that links it to integrins (Mitra et al., 2005; Schaller, 2010). FAK is activated by integrin, growth
factor, and G protein–linked and cytokine stimuli that increase FAK tyrosine phosphorylation at tyrosine (Y) 397, Y576, Y861,
and Y925 and link FAK to MAPK activation (Schlaepfer and Mitra, 2004). Global or EC-specific FAK knockout results in
embryonic lethality associated with vascular defects (Ilic et al., 2003; Shen et al., 2005; Braren et al., 2006). A p53 tumor suppressor–
dependent block in cell proliferation is associated with the FAK knockout phenotype (Lim et al., 2008). However, the presence of the FAK-related Pyk2 PTK in cells without FAK reduces effects of FAK loss on p53 and angiogenesis (Weis et al., 2008; Lim et al., 2010b). Although recent studies using condi
Fak knockout in ECs prevent tumor-induced vascular permeability (Lee et al., 2010) and angiogenesis (Tavora et al.,
2010), the in vivo signaling connections for FAK in mediating these effects remains unclear.

Knock in mouse models have revealed that FAK activity is critical for developmental vasculogenesis (Lim et al., 2010a; Zhao et al., 2010). Additionally, pharmacological FAK inhibition suppresses tumor- and growth factor–stimulated angiogenesis (Roberts et al., 2008; Weis et al., 2008). FAK kinase-dead (KD) knockin mutation is embryonic lethal, and FAK-KD fibroblasts exhibit defects in cell polarity and motility but not cell prol
feration (Lim et al., 2010a). FAK-KD protects primary ECs from increased apoptosis through a mechanism that involves FAK-FERM–mediated nuclear localization, binding to p53, and enhancement of murine double minute 2 (Mdm2) E3 ligase–
dependent p53 ubiquitination and proteasome degradation (Lim et al., 2010b). Thus, nuclear FAK can regulate cell prol
feration and survival by facilitating p53 turnover in a kinase-independent manner. As nuclear-localized FAK contributes to
FAK activity controls VCAM-1 expression during mouse development

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VCAM-1 expression in the developing mouse embryo allantois promotes binding to α4β1 integrin on chorion membrane, and this union facilitates chorioallantoic fusion (Inman and Downs, 2007). FAK-KD knockin mutation results in lethality at embryonic day 9.5 (E9.5; Lim et al., 2010a), with embryos exhibiting an enlarged and unfused allantois (Fig. 2 A). FAK-KD administration to pregnant mice also results in E9.5 embryo lethality with defective allantois structures (Fig. 2 B). FAK-KD embryos fail to form somites, exhibit gross defects in head and heart structures, and form a rudimentary gut cavity (Fig. 2 C). Comparisons of mRNAs within FAK–wild type (WT) and FAK-KD embryos by array analyses revealed several targets that differed in expression greater than twofold. In particular, VCAM-1 mRNA levels were reduced 7.4 fold in FAK-KD embryos (Table 1). Gene ontology analyses of the mRNA array data revealed functional annotated differences that connect FAK activity to a leukocyte extravasa
tion signaling group (Fig. S3). Other connections to endocytosis,
FAK inhibition prevents cytokine-stimulated VCAM-1 expression in human ECs

Regulation of mouse and human VCAM-1 expression share both similarities and mechanistic differences (Minami and Aird, 2001). To determine whether FAK is also important in human TNF-α–induced human VCAM-1 expression, signaling experiments were performed with human umbilical vein ECs (HUVECs). FAK-I treatment prevented TNF-α–mediated VCAM-1 expression in a dose-dependent manner with an IC₅₀ of ~0.5 µM that paralleled inhibition of FAK Y397 phosphorylation (Fig. 3 A). This effect was a result in part of transcriptional effects, as FAK-I significantly inhibited TNF-α–induced VCAM-1 promoter luciferase activity (Fig. 3 B). Knockdown of FAK expression also significantly inhibited TNF-α–induced VCAM-1 protein and mRNA expression (Fig. 3, C and D). As FAK-I treatment of HUVECs also prevented IL-1β–stimulated VCAM-1 expression (Fig. 3 E), our experiments show that FAK functions as a common signaling component regulating inflammatory cytokine-stimulated VCAM-1 expression.

FAK facilitates TNF-α–induced MAPK but not NF-κB activation

TNF-α activates extracellular-regulated kinase (ERK) MAPKs, JNK MAPKs, and p38 MAPKs as well as NF-κB to regulate gene expression (Karlin and Gallagher, 2009). To determine the signaling consequences of FAK inhibition, comparisons of FAK-WT and FAK-KD MEFs or DMSO (control) and FAK-I–treated HUVECs were performed (Fig. 4). TNF-α rapidly and equally promoted NF-κB p5336 phosphorylation in both control and FAK-I–treated cells (Fig. 4, A and B). Regulatory IkBα was rapidly degraded after TNF-α addition (Fig. 4, A and B), and electrophoretic mobility shift assays (EMSAs) revealed equivalent TNF-α–stimulated NF-κB DNA binding in FAK-KD MEFs and FAK-I–treated HUVECs compared with FAK-WT and DMSO-treated controls (Fig. S4). In vivo, FAK-I significantly inhibited TNF-α–induced FAK Y397 phosphorylation but did not alter TNF-α–stimulated NF-κB DNA binding in mouse lung lysates (Figs. 4 C and S5). Despite the importance of NF-κB in...
MEF reconstitution experiments have verified the importance of FAK activity and Y397 FAK phosphorylation in facilitating TNF-α-stimulated ERK/MAPK activation and IL-6 gene expression (Schlaepfer et al., 2007), our experiments evaluating ERK/MAPK and VCAM-1 extend this TNF-α and FAK signaling connection to HUVECs and to mice.

FAK inhibition alters GATA4 transcription factor levels

To establish the signaling linkage between FAK, MAPK, and the regulation of VCAM-1 transcription, rescue assays were performed by transient transfection of FAK-WT or activators of MAPKs (constitutively active MEK1, MKK4, or MEKK1) in FAK-KD MEFs or HUVECs treated with FAK-I. Unexpectedly, neither FAK-WT nor active MAPK alone or in combination was able to increase VCAM-1 expression upon TNF-α stimulation (unpublished data). As VCAM-1 production is transcriptionally regulated, we investigated whether FAK inhibition may alter the profile of VCAM-1–associated transcription factors in cells (Ahmad et al., 1998; Molkentin, 2000; Minami and Aird, 2001). Although genetic or pharmacological FAK inhibition did not prevent NF-κB activation upon TNF-α stimulation, promoting TNF-α–induced VCAM-1 gene transcription. (Karin and Gallagher, 2009), FAK inhibition did not detectably alter NF-κB activation.

Instead, comparisons of WT and FAK-KD MEFs or FAK-I–treated HUVECs revealed that FAK-I reduced TNF-α–stimulated JNK/MAPK and ERK/MAPK activation at 5 and 15 min (Fig. 4, A and B). FAK-I treatment results in the elevation of total FAK expression but not pY397 FAK phosphorylation in HUVECs (Fig. 4 B). In lung lysates, pretreatment of mice with FAK-I significantly prevented TNF-α–induced ERK/MAPK activation in vivo (Figs. 4 C and S5), and a previous study showed that pharmacological ERK inhibition prevented TNF-α–induced VCAM-1 expression in ECs (Fitau et al., 2006). Our results support the conclusion that FAK activity facilitates TNF-α signaling to MAPKs needed for VCAM-1 expression. This role for FAK may be related to cross-talk between integrin and cytokine signaling pathways, as TNF-α signaling to ERK/MAPK (Fig. 4 D), induction of VCAM-1 expression (Fig. 4 E), and FAK Y397 phosphorylation (Fig. 4, D and E) are prevented when MEFs are deprived of matrix-mediated cell adhesion. Serum addition limited suspension-induced cell death, as determined by annexin V staining (unpublished data). As FAK−/− MEF reconstitution experiments have verified the importance of FAK activity and Y397 FAK phosphorylation in facilitating TNF-α–stimulated ERK/MAPK activation and IL-6 gene expression (Schlaepfer et al., 2007), our experiments evaluating ERK/MAPK and VCAM-1 extend this TNF-α and FAK signaling connection to HUVECs and to mice.

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and resulted in an ~90% loss of GATA4 within 6 h without effects on GATA6 (Fig. 5 B). Interestingly, these FAK-I–triggered changes in GATA4 expression occurred without alterations in GATA4 mRNA levels (Fig. 5 C). Thus, inhibition of FAK selectively affects GATA4 protein but not mRNA levels in cells.

**FAK and GATA4 rescue TNF-α–induced VCAM-1 expression in FAK-KD MEFs**

To determine the significance of GATA4 loss upon FAK inhibition, FAK-WT and GATA4 alone or in combination were transfected into FAK-KD MEFs, and the effect on VCAM-1 production was evaluated in the presence or absence of TNF-α stimulation (Fig. 5 D). Notably, only the combination of FAK-WT and GATA4 with TNF-α stimulation was able to promote VCAM-1 expression. Additionally, siRNA knockdown of GATA4 in FAK-WT MEFs significantly reduced TNF-α–induced VCAM-1 protein and mRNA levels (Fig. 5, E and F). Quantitative PCR (Q-PCR) was used to confirm that GATA4 and VCAM-1 mRNA were significantly suppressed after TNF-α stimulation by siRNA to GATA4 compared with control siRNA transfection (Fig. 5, G and H). In vivo, GATA4 and VCAM-1 protein levels were increased in heart lysates after TNF-α stimulation, and this was significantly inhibited by FAK-I administration (Fig. 4), immunoblotting revealed the absence of GATA4 expression in FAK-KD MEFs, whereas GATA6 was equally expressed in FAK-WT and FAK-KD MEFs (Fig. 5 A). Further, FAK-I treatment of cells also resulted in the selective loss of GATA4 but not GATA6 expression by >50% within 3 h (not depicted) and resulted in an ~90% loss of GATA4 within 6 h without effects on GATA6 (Fig. 5 B). Interestingly, these FAK-I–triggered changes in GATA4 expression occurred without alterations in GATA4 mRNA levels (Fig. 5 C). Thus, inhibition of FAK selectively affects GATA4 protein but not mRNA levels in cells.

### Table 1. Differences in target mRNAs from FAK-WT and FAK-KD embryos during development

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Fold reduction (WT/KD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropomodulin-1</td>
<td>13.1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>7.4</td>
</tr>
<tr>
<td>Caveolin-1</td>
<td>6.4</td>
</tr>
<tr>
<td>Robo-4</td>
<td>4.9</td>
</tr>
<tr>
<td>Rgs5</td>
<td>4.7</td>
</tr>
<tr>
<td>CD31</td>
<td>3.6</td>
</tr>
<tr>
<td>WAS</td>
<td>4.3</td>
</tr>
<tr>
<td>ESAM-1</td>
<td>2.7</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>2.6</td>
</tr>
<tr>
<td>α-SMA</td>
<td>2.3</td>
</tr>
<tr>
<td>SEMA6B</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Illumina MouseWG-6 v2.0 Expression BeadChip analyses (45,200 targets) were performed. Selected mRNAs with greater than twofold change are shown. WAS, Wiskott–Aldrich syndrome; ESAM-1, endothelial selective adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; α-SMA, α-smooth muscle actin.
or ubiquitination and proteasome degradation (Brewer and Pizzey, 2006). The loss of GATA4 expression by FAK-I treatment was reversed by addition of a cell-permeable proteasome inhibitor (MG132; Fig. 6 A), supporting the notion that FAK inhibition may promote enhanced GATA4 degradation. Notably, FAK can also promote p53 tumor suppressor degradation to mice (Fig. 5 I). Together, these results support the importance of GATA4 in TNF-α-induced VCAM-1 expression and suggest that loss of GATA4 associated with FAK inhibition prevents VCAM-1 production.

Regulation of GATA4 protein levels can occur through various posttranslational mechanisms, including phosphorylation or ubiquitination and proteasome degradation (Brewer and Pizzey, 2006). The loss of GATA4 expression by FAK-I treatment was reversed by addition of a cell-permeable proteasome inhibitor (MG132; Fig. 6 A), supporting the notion that FAK inhibition may promote enhanced GATA4 degradation. Notably, FAK can also promote p53 tumor suppressor degradation.
FAK-FERM binds GATA4 and enhances GATA4 ubiquitination

Localization of FAK to integrin-enriched sites of adhesion occurs via the FAK C-terminal domain, whereas the FAK-FERM domain can target FAK to the nucleus (Lim et al., 2008). Adeoviral (Ad)-mediated overexpression experiments in human 293T cells revealed that FAK-WT, FAK-KD, and FAK-FERM but not FAK-CT associated with endogenous GATA4 by coimmunoprecipitation (Fig. 6 D). In vitro translation of various FAK constructs combined with binding assays using GST fusion proteins of GATA4 N-terminal and CT domains revealed direct binding between FAK-WT, FAK-KD, and FAK-FERM with the N-terminal domain of GATA4 (Fig. 6 E). No binding was detected between the FAK kinase domain and GATA4.

(Fig. 5. FAK inhibition decreases GATA4 levels needed for TNF-α–induced VCAM-1 expression. (A) Steady-state GATA4 and GATA6 levels in FAK-WT and FAK-KD MEFs, as determined by immunoblotting with actin as a control. (B) FAK-WT MEFs treated with DMSO or FAK-I (1 µM PF271), 6 h and lysates blotted for GATA4 or GATA6. Anti-GAPDH blotting is shown as loading controls. (C) GATA4 mRNA levels to GAPDH were determined by Q-PCR (±SD; n = 3) in experiments, as described in B. (D) Rescue of TNF-α–induced VCAM-1 expression in FAK-KD MEFs by combined FAK-WT and GATA4 expression. Combinations of GFP-FAK and GATA4 were transfected into FAK-KD MEFs. After 24 h, cells were stimulated with 10 ng/ml TNF-α, as indicated, and FAK, VCAM-1, and GATA4 immunoblotting was performed at 48 h. (E) FAK-WT MEFs were transfected with Scr or GATA4 (G4) siRNA and, after 48 h, stimulated with TNF-α (10 ng/ml, 6 h), and immunoblotting was performed for VCAM-1 and GATA4. Anti-actin and anti-GAPDH blotting are shown as loading controls. (F) Densitometry analyses of VCAM-1 protein levels relative to actin, as described in E. (G and H) MEFs were transfected with Scr or GATA4 siRNA and stimulated with TNF-α, as described in E. GATA4 (G) or VCAM-1 (H) mRNA levels to GAPDH were determined by qRT-PCR (±SD; n = 3; ****, P < 0.0001). (I) PBS or TNF-α [0.02 mg/kg] was tail vein injected into mice, and, after 6 h, heart lysates were analyzed by immunoblotting for VCAM-1, GATA4, py397 FAK, total FAK, and actin. Where indicated, FAK-I (100 mg/kg, PND-1186) was administered 3 h before starting experiments. Data are mean densitometry values relative to actin (±SD; n = 2; *, P < 0.05; ***, P < 0.001).

(Lim et al., 2008). Cell stress triggers FAK nuclear localization, a nuclear FAK–p53 complex, and FAK-enhanced Mdm2 E3 ligase–dependent ubiquitination and degradation of p53 (Lim et al., 2008). In FAK-KD MEFs, FAK is localized to sites of adhesion as well as to the nucleus (Lim et al., 2010a). Biochemical separation of FAK-WT and FAK-KD MEFs into cytosolic and nuclear fractions revealed elevated levels of FAK-KD in the nucleus (Fig. 6 B). Importantly, FAK-I treatment of cells could trigger enhanced FAK nuclear accumulation within 3–6 h (Fig. 6 C). Interestingly, the timing of FAK nuclear localization upon FAK-I addition parallels the time course of GATA4 loss (Figs. 5 B and 6 C). Together, these results show that FAK inhibition promotes increased FAK nuclear accumulation–correlated loss of GATA4 protein.

Figure 5. FAK inhibition decreases GATA4 levels needed for TNF-α–induced VCAM-1 expression. (A) Steady-state GATA4 and GATA6 levels in FAK-WT and FAK-KD MEFs, as determined by immunoblotting with actin as a control. (B) FAK-WT MEFs treated with DMSO or FAK-I (1 µM PF271), 6 h and lysates blotted for GATA4 or GATA6. Anti-GAPDH blotting is shown as loading controls. (C) GATA4 mRNA levels to GAPDH were determined by Q-PCR (±SD; n = 3) in experiments, as described in B. (D) Rescue of TNF-α–induced VCAM-1 expression in FAK-KD MEFs by combined FAK-WT and GATA4 expression. Combinations of GFP-FAK and GATA4 were transfected into FAK-KD MEFs. After 24 h, cells were stimulated with 10 ng/ml TNF-α, as indicated, and FAK, VCAM-1, and GATA4 immunoblotting was performed at 48 h. (E) FAK-WT MEFs were transfected with Scr or GATA4 (G4) siRNA and, after 48 h, stimulated with TNF-α (10 ng/ml, 6 h), and immunoblotting was performed for VCAM-1 and GATA4. Anti-actin and anti-GAPDH blotting are shown as loading controls. (F) Densitometry analyses of VCAM-1 protein levels relative to actin, as described in E. (G and H) MEFs were transfected with Scr or GATA4 siRNA and stimulated with TNF-α, as described in E. GATA4 (G) or VCAM-1 (H) mRNA levels to GAPDH were determined by qRT-PCR (±SD; n = 3; ****, P < 0.0001). (I) PBS or TNF-α [0.02 mg/kg] was tail vein injected into mice, and, after 6 h, heart lysates were analyzed by immunoblotting for VCAM-1, GATA4, py397 FAK, total FAK, and actin. Where indicated, FAK-I (100 mg/kg, PND-1186) was administered 3 h before starting experiments. Data are mean densitometry values relative to actin (±SD; n = 2; *, P < 0.05; ***, P < 0.001).
Notably, overexpression of FAK-WT, FAK-KD, or FAK-FERM increased GATA4 polyubiquitination (Fig. 6 F). These experiments were performed in the presence of MG132 proteasome inhibitor to stabilize and detect polyubiquitylated GATA4 by immunoblotting. Interestingly, treatment of MEFs with FAK-I enhanced GATA4 ubiquitination, and this occurred equally in FAK-WT and Mdm2−/− MEFs (Fig. 7 A). As GATA4 ubiquitination is mediated in part by the cochaperone/ubiquitin ligase CHIP in response to cellular stress (Dai et al., 2003; Kobayashi et al., 2007), CHIP−/− MEFs were treated with MG132 (40 µM, 3 h). Whole-cell lysates were analyzed for expression of FAK or actin (left), and flag tag immunoprecipitates (antibody coupled to beads) were evaluated by anti-ubiquitin (Ub) and GATA4 immunoblotting. Interestingly, treatment of MEFs with FAK-I enhanced GATA4 ubiquitination, and this occurred equally in FAK-WT and Mdm2−/− MEFs (Fig. 7 A). As GATA4 ubiquitination is mediated in part by the cochaperone/ubiquitin ligase CHIP in response to cellular stress (Dai et al., 2003; Kobayashi et al., 2007), CHIP−/− MEFs were treated with MG132 (40 µM, 3 h). Whole-cell lysates were analyzed for expression of FAK or actin (left), and flag tag immunoprecipitates (antibody coupled to beads) were evaluated by anti-ubiquitin (Ub) and GATA4 immunoblotting.

Discussion

Induced VCAM-1 expression contributes to vascular inflammation (Libby, 2002). Here, we show through both genetic and pharmacological experimental results that FAK activity is essential in promoting TNF-α–induced VCAM-1 expression. These experiments define a new role for nuclear FAK in the regulation of inflammation-induced gene expression. Our results support a model (Fig. 8) whereby under normal signaling conditions, FAK functions in the cytoplasm to facilitate GATA4 phosphorylation and promotes the turnover of GATA4 protein as mechanisms limiting TNF-α–induced VCAM-1 expression.
Upon genetic or pharmacological FAK inhibition, FAK accumulates in the nucleus and acts to enhance GATA4 polyubiquitination. Loss of GATA4 prevents TNF-α-stimulated VCAM-1 expression, and this block cannot be rescued by constitutive MAPK activation. The FAK-FERM domain promotes FAK nuclear localization and direct binding to GATA4 and acts as a scaffold to enhance CHIP-dependent ubiquitination, leading to GATA4 degradation. These experiments reveal a novel anti-inflammatory role for nuclear-localized and kinase-inhibited FAK in limiting VCAM-1 production through the modulation of GATA4 activation and turnover.

Our experiments also expand the role for FAK in mediating inflammatory signals. FAK activity promotes TNF-α-induced IL-6 expression (Funakoshi-Tago et al., 2003; Schlaepfer et al., 2007), and FAK-I treatment of mice prevents orthotopic breast tumor–associated leukocyte infiltration and tumor-associated splenomegaly (Walsh et al., 2010). As conditional deletion of FAK expression prevents macrophage or neutrophil cell migration (Owen et al., 2007), anti-inflammatory effects of FAK-I administration may result from the intrinsic inhibition of macrophage motility as well as stromal-associated mediators such as changes in VCAM-1 gene expression. As FAK-Is are being evaluated in human clinical trials as agents blocking tumor growth and angiogenesis (Schultze and Fiedler, 2010), our experiments evaluating VCAM-1 and a recent study of dominant-negative FAK inhibition preventing EC-associated E-selectin up-regulation (Hiratsuka et al., 2011) support a greater need to understand the role of FAK inhibition in altering inflammatory-associated vascular responses.

A previous study showed that FAK is catalytically activated by TNF-α stimulation of cells (Schlaepfer et al., 2007). Although other PTKs are associated with TNF-α signaling, past studies have linked PTK activity to TNF-α–induced NF-κB activation (Huang et al., 2003; Takada and Aggarwal, 2004). In contrast, FAK inhibition selectively reduced TNF-α activation of MAPKs but had no effect on NF-κB activation. Importantly, it is signaling from multiple pathways that combine to activate AP-1, NF-κB, and GATA transcription factors necessary for...
binding to cell surface receptors triggers intracellular signaling cascade activation of MAPKs and NF-κB. This leads to alterations in gene transcription of targets such as VCAM-1 that is regulated in part by combined effects of AP-1, GATA, and NF-κB transcription factors. Inhibition of FAK prevents TNF-α–induced MAPK activation and the inhibition of GATA4 Ser105 phosphorylation. Inhibited FAK (FAK-KD) accumulates in the nucleus, binds directly to GATA4, and promotes increased GATA4 ubiquitination and proteasomal degradation via interactions with the CHIP E3 ubiquitin ligase. Impairment in both FAK-mediated MAPK activation and GATA4 stability prevent cytokine-stimulated VCAM-1 transcription and reveal novel anti-inflammatory effects of FAK inhibition.

VCAM-1 transcriptional regulation (Karin and Gallagher, 2009). Notably, GATA4 is activated by MAPK phosphorylation at S105 (van Berlo et al., 2011), and β1 integrin signaling through MAPK contributes to GATA4 nuclear translocation and activation (Liu et al., 2009). As FAK is the major PTK activated by integrins and we find that matrix adhesion is required for efficient TNF-α–stimulated ERK/MAPK activation and VCAM-1 production, our results support the importance of FAK activity in mediating cross-talk between integrins and cytokine receptor signaling pathways.

Although FAK inhibition prevents TNF-α–induced GATA4 S105 phosphorylation, interpretations are complicated by a corresponding reduction in GATA4 protein levels. We find that genetic or pharmacological FAK inhibition triggers full-length corresponding reduction in GATA4 protein levels. We find that S105 phosphorylation, interpretations are complicated by a

Mainly, VCAM-1 is a marker of endothelial activation and a major adhesion molecule for leukocytes. Its expression is induced by cytokines such as TNF-α, through the activation of transcription factors such as NF-κB and AP-1. GATA4, a transcription factor, is activated by MAPK phosphorylation at Ser105, and its activation is required for VCAM-1 expression.

In summary, our genetic and pharmacological inhibition experiments reveal that FAK activity regulates VCAM-1 gene expression during development and expand the role of FAK signaling as an essential mediator of TNF-α–induced VCAM-1 production. The regulation of GATA4 by nuclear FAK provides a novel twist to the anti-inflammatory effects of FAK inhibition.

### Materials and methods

#### Mice

Mice

FAK-KD knockin mice containing a point mutation within the FAK kinase domain (changing the codon for Lys454 to Arg) were generated by homologous recombination, as previously described (Lim et al., 2010a), maintained as a heterozygous population, and backcrossed to a C57Bl6 background (The Jackson Laboratory) for 10 generations. Mouse experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee, and mice were maintained in accordance with Association for Assessment and Accreditation of Laboratory Care International–approved guidelines. Early-passage FAK-WT and FAK-KD MEfS were generated, immortalized by hTERT expression, and maintained on 0.1% gelatin-coated dishes, as previously described (Lim et al., 2010a). FAK-WT and FAK-KD ECs were isolated, immortalized by large T antigen expression (Addgene), and maintained as previously described (Zhao et al., 2010). HUEC1, 293T, and Mdm2+/p53−/− fibroblasts were used as previously described (Lim et al., 2008). CHIP+/− fibroblasts were obtained from C. Patterson (University of North Carolina, Chapel Hill, NC; Dai et al., 2003). Cells were maintained in DME with 10% FBS, nonessential amino acids for MEM, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 µg/ml streptomycin. Cell culture–based experiments were performed with semi-confluent cells in growth media. Mouse GATA4 cDNA was obtained from P. Mellon (University of California San Diego, La Jolla, CA; Lawson et al., 1996) and was subcloned into p3XFlagCMV7.1 (Sigma-Aldrich). GST fusion N-terminal (aa 1–260; ends with first DNA-binding zinc finger motif) and CT (aa 255–440; starts with second zinc finger motif) GATA4 constructs (pGEV21) were obtained from R. Viger (Centre hospitalier de l’Université de Montréal).
Laval Research Centre, Ste-Foy, Quebec, Canada; Tremblay and Viger, 2003). Ad tetracycline transactivator (TA) was added to induce various Ad-FAK constructs and the indicated HA- or Myc-tagged full-length FAK-WT, and FAK-KD, FAK-FERM (aa 1–402, N-terminal fragment), and FAK-CT (aa 683–1,024, containing focal adhesion targeting domain) constructs were used as previously described (Lim et al., 2008; Schlaepfer et al., 2007). LentiViral scrambled (Scr) and anti–human FAK short hairpin RNAs were used as previously described (Lim et al., 2008). Mouse CHIP cDNA was purchased (Thermo Fisher Scientific), and PCR was used to amplify the coding sequence and subclone into pcDNA3.1 3XHA for expression as an HA-tagged protein.

Antibodies and reagents
Anti-FAK (clone 4.47), anti–mouse VCAM-1 (M2/K2), and glycerolaldheyde 3-phosphate dehydrogenase [GAPDH; 374] were purchased from Milli-Port. Anti-mouse VCAM-1 [112702] for blotting was purchased from R&D Systems. Anti–p-Y397 FAK (44625G), anti–p-Y576 FAK (70013), anti-p-ERK [pT202/pY204; 44654G], and anti-p-GATA4 [pS105; 44–948] were purchased from Life Technologies. Anti–NF-kB p55/63 [93H11] and anti–p-JNK [pT183/pY185; 9251] were purchased from Cell Signaling Technology. Anti–β-actin [AC-17], anti-talin [B8d4], and anti-Mag [M4] tag were purchased from Sigma-Aldrich. Anti–human VCAM-1 [H2767], anti-lcbs [C21], anti-GATA4 [G-4], anti-GATA6 [H927], anti-JNK2 [D-2], and anti-ERK2 [12A4] were purchased from Santa Cruz Biotechnology, Inc. Anti–HA [16B12] and anti-Myc tag [PE10] were purchased from Covance. Anti–terminal ADP ribose polymerase (PARP; clone 1801), anti–anti-D (ME13), and anti–anti-C (ME12) were purchased from BD. Anti–poly ADP ribose polymerase (PARP; clone 42), anti-CD31 (MEC13.3), and anti–ubiquitin (Fk2) were purchased from Enzo Life Sciences, and anti–GAPDH was purchased from GeneTex Inc. (GT2392). Recombinant TNF-α (human and mouse) and IL-β [human] were purchased from R&D Systems. Bovine plasma fibrinectin (FN) was purchased from Sigma-Aldrich. MG132 was purchased from EMD. Mouse GATA4 ON-TARGETplus SMART pool siRNA [D040759-01-0005] and ON-TARGETplus Si Control (Scr) siRNA [D001810-01] were purchased from Thermo Fisher Scientific. 100 pmol siRNA was used to transfect WT MEFs using Lipofectamine 2000 (Life Technologies). After 48 h, target knockdown was confirmed by immunoblotting.

FAK-Is and in vivo TNF-α signaling
FAK1-PF-271 was synthesized by Liviana Corp., as described in patent materials (Roberts et al., 2008), solubilized in DMSO, and used in experiments with cultured cells at 1 µM. FAKI-PD-11886 (Poniard Pharmaceuticals) was used for in vivo mouse experiments (Tianjoni et al., 2010; Walsh et al., 2010), solubilized in water, and administered twice daily via oral gavage in phosphate buffer (0.02 mg/kg in 100 µl PBS) or PBS was tail vein injected into 2010), solubilized in water, and administered twice daily via oral gavage in phosphate buffer (0.02 mg/kg in 100 µl PBS) or PBS was tail vein injected into

Biochemical analyses
FAK-I (1 µM PF271) was added to growing cells for 1 h before TNF-α addition (10 ng/ml) unless otherwise indicated. For reporter assays, growing cells were treated with 0.06% trypsin and 2 mM EDTA in PBS (2.5 min at 37°C), centrifuged, resuspended in DME with 10% FBS, enumerated (Vi-CELL XR; Beckman Coulter), and held at 37°C (2 × 10^5 cells/ml) for 30 min. FN-coated (10 µg/ml in PBS overnight) plate was used with 1% BSA in PBS for 30 min and preheated to 37°C before cell replating (1 h) followed by TNF-α addition for 1.5 min to 6 h. Cell apoptosis was determined after 7 h by collecting cells with trypsin and staining for Annexin V/propidium iodide with a FACSCalibur; BD) using allophycocyanin–annexin V and 7-actinomycin D staining (BD). Tissue and cultured cell protein lysates were prepared in cell extraction buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 10% glycerol. For immunoprecipitation and GST binding analyses, lysates were subjected to diluted twofold in HNTG buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1% glycerol) and incubated with 1 µg of FLAG-GATA4 or glutathione agarose beads (Sigma-Aldrich) for 6 h at 4°C. Cell pellets were washed at 4°C in 1% Triton X-100–only extraction buffer followed by washes with HNTG buffer and resolved by SDS-PAGE. Densitometry of immunoblotting images was performed using ImageJ (v.1.44; National Institutes of Health).

To evaluate ubiquitin incorporation into GATA4, experiments were performed in human HEK293 or normal Mdm2−/− or CHIP−/− MEFs. MEFs were infected with 50 plaque-forming unit (pfu)/cell Ad-FAK or Ad-FAK-FERM constructs and 5 pfu/cell Ad-TA. Controls were performed with 50 pfu/cell Ad-TA. For transient transfection, 293T or MEFs using jetPEI (Polyplus Transfection), 1 µg Flag-GATA4, 0.5 µg HA-ubiquitin, or His-ubiquitin, and 5 µg FAK-FERM plasmid DNA were used. 40 µM MG132 was added 3 h before cell lysis for ubiquitin analyses. For ubiquitin conjugate purification, transfected cells (24 h) were lysed with 8 M urea in phosphate buffer (0.1 M NaH2PO4,10 mM Tris-HCl, pH 8.0, and 20 mM imidazole) at room temperature. His-ubiquitin–conjugated proteins were bound to a nickel agarose column (Ni-NTA; Qiagen) and washed with 8 M urea phosphate buffer, pH 6.5, and proteins were eluted in 8 M urea phosphate buffer at pH 4.5.

For fractionation experiments, cells were lysed with Cyt buffer (10 mM Tris, pH 7.5, 0.05% NP-40, 3 mM MgCl2, 100 mM NaCl, 1 mM EGTA, 20 µg/ml aprotinin, 1 mM orthovanadate, and 10 µg/ml leupeptin), scraped loaded into tubes, incubated for 5 min at 4°C, and spun at 800 g at 4°C (5 min), and cytosolic supernatants were collected. Cell pellets were further washed with Cyt buffer, purified nuclei were resuspended in cell extraction buffer and spun at 16,000 g for 15 min, and the supernatant was collected as the nuclear fraction. Samples were separated by SDS-PAGE and immunoblotted for GAPDH and PARP as cytoplasmic and nuclear markers, respectively.

For direct binding assays, prey proteins were in vitro translated using the TNT transcription–translation system (Promega). Various pCDNA3 FAK expression constructs (1 µl) were translated in a mixture containing biotin-labeled Lys and diluted 50 fold into binding buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, and 1% Triton X-100). Purified GST-GATA4 proteins were prebound to glutathione agarose beads, incubated in vitro translated prey for 2 h at 4°C, and washed three times in binding buffer, and the bound prey were detected by streptavidin-HRP immunobluccling.
VCAM-1 promoter assay

HUVeCs were cotransfected using jetPEHUVeC (PolyPlus Transfection) with Renilla luciferase and promoterless pGL3 luciferase control (Promega) or pGL3 containing 1.8 kb of the human VCAM-1 promoter (−1,716 to 119) from W. Aird (Beth Israel Deaconess Medical Center, Boston, MA; Minami and Aird, 2001). After 18 h, 10 ng/ml TNF-α was added in the presence or absence of 1 μM FAK-I, and luciferase activity was measured with the dual assay kit (Promega) after 6 h.

NF-kB EMSA

Cells were scraped into 1 ml cold PBS, pelleted by centrifugation (1,500 g for 10 min), and resuspended in 160 μl of buffer A (50 mM KCl, 25 mM Hepes, pH 7.8, 10 μg/ml leupeptin, 20 μg/ml apronitin, 125 mM DTT, and 1 mM PMSF) on ice for 15 min. 40 μl of 2.5% NP-40 was added, vortexed, and centrifuged (12,000 g for 5 min). Pellets were washed with cold buffer A, resuspended in 40 μl of buffer A with 500 mM KCl, frequently vortexed for 20 min, and centrifuged (12,000 g for 5 min). The supernatant protein concentration was determined by a Bradford assay (Bio-Rad Laboratories) and stored frozen (−70°C) as a nuclear extract. For tissue extracts, 3 g of lung tissue was minced with a razor blade, and single-cell suspensions were prepared by Dounce homogenization in 4 ml of buffer A. Nuclear extracts were prepared as previously described. Double-stranded NF-κB oligonucleotides (Promega) were labeled with [γ-32P]ATP (PerkinElmer) using DNA T4 polynucleotide kinase (New England Biolabs, Inc.), and unincorporated nucleotides were removed using a G-25 spin column (GE Healthcare). Labeled probes (∼20,000 cpm per 1 μmol) were incubated with 4 μg of nuclear extract for 30 min at room temperature in gel shift binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 40 mM KCl) containing 100 μg/ml poly(deoxyinosinic-deoxycytidylic) acid and 10% glycerol. Addition of 100-fold excess of unlabelled oligonucleotide probe (10 μm) was used to verify binding specificity. Samples were resolved by 4% native PAGE, and binding was visualized by autoradiography.

Immunofluorescent staining

Tissues were sectioned (6 μm; CM1950; Leica), fixed in cold acetone (10 min), rehydrated in PBS containing 0.5% BSA (5 min), and blocked with 1.25% normal goat serum in PBS (30 min at room temperature). Samples were incubated with anti–FAK pY576 (1:50) or anti–VCAM-1 (M-K/2; 1:50) and anti-CD31 (1:300) overnight at 4°C. Alexa Fluor 488 goat anti–rat and Alexa Fluor 594 goat anti–rabbit secondary antibodies (1:500, 30 min at room temperature; Life Technologies). For VCAM-1 and CD31 staining, anti-VCAM-1 was labeled with Dylight 594 (red) by using a Dylight microscale antibody labeling kit (Thermo Fisher Scientific). Samples were incubated with fluorescein isothiocyanate cytochrome (1:50) and Dylight 488–conjugated nitro blue tetrazolium (1:25) overnight. Slides were mounted in VECTASHIELD (H-1000; Vector Laboratories), and images were acquired at room temperature sequentially using a mercury lamp source, multiblend dichroic, single-band exciter, and single-band emitter filter sets (Chroma Technology Corp.) on dual filter wheels, a spinning-disk confocal microscope (IX81; Olympus) at 60X (PlanApo, NA 1.42), and an OrcaER camera (Hamamatsu Photonics) controlled by Slidebook software (v 5.0). Files were deconvolved, pseudo-colored, and contrast adjusted using Photoshop CS3. The degree of association exhibited by patterns of fluorescence was measured on a pixel-by-pixel basis and calculated as a Pearson’s correlation coefficient using the Cell Profiler measure correlations module (v2.0; Broad Institute). A value of 0 indicates no overlap, and a value of 1 corresponds to 100% colocalization. Adhesion size (pixels) and number within a cell were determined using Cell Profiler using a pipeline to threshold images and reduce background fluorescence staining.

Statistical analyses

Significance between experimental groups was determined by one-way analysis of variance with a Tukey’s post hoc test using Prism software (v3.0b; GraphPad Software).

Online supplemental material

Fig. S1 shows quantitative analyses of TNF-α–stimulated VCAM-1 protein expression in mouse lung tissue and the inhibition of VCAM-1 by oral FAK-I (PND-1186) pretreatment. Fig. S2 shows visualization of TNF-α–stimulated FAK activation and VCAM-1 expression within heart ECs by indirect immunofluorescent staining. Fig. S3 shows gene ontology and signaling network analyses of mRNA array data from FAK-WT and FAK-KD embryos. Fig. S4 shows EMSAs in MEFs and HUVeCs whereby FAK inhibition (FAK1 PF271) does not block TNF-α–stimulated NF-κB activation. Fig. S5 shows analyses of lung lysates whereby pharmacological FAK inhibition (PND-1186) blocks TNF-α–induced ERK/MAPK but not NF-κB activation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201109067/DC1.

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Figure S1. **TNF-α–stimulated VCAM-1 expression in lung tissue is dependent on FAK activity.** (A) Experimental timeline. Oral administration of FAK-I (100 mg/kg, PND-1186) was administered 3 h before TNF-α tail vein injection (0.02 mg/kg), and tissues were collected at 6 h for immunoblotting after TNF-α stimulation. (B) Lung lysates were analyzed for VCAM-1, pY397 FAK, and total FAK blotting. Actin was used as loading controls (Con). (C) Lung-associated VCAM-1 expression or FAK activation (pY397) was measured by densitometry, and ratios to actin and total FAK were determined. Values are means ± SD; **, P < 0.001; ****, P < 0.0001) from six mice, representing two independent experiments.
Figure S2. Visualization of TNF-α–stimulated FAK activation and VCAM-1 expression within heart ECs. In vivo signaling assays were performed with C57BL6 mice pretreated with vehicle or FAK-I (100 mg/kg, PND-1186) by tail vein injection of recombinant TNF-α (0.02 mg/kg) or PBS. Hearts were isolated after 6 h, and frozen sections were analyzed by combined indirect immunofluorescent staining. (A) Staining for ECs (CD31) and activated FAK (pY576). In the merged images, yellow staining indicates sites of pY576 FAK and CD31 costaining. (B) Staining for ECs (CD31) and VCAM-1. In the merged images, yellow staining indicates sites of VCAM-1 and CD31 costaining. Bars, 20 µm.
Figure S3.  Gene ontology and signaling network analyses of mRNA array data from FAK-WT and FAK-KD embryos. (A) Shown is Ingenuity Pathway Analysis (Ingenuity Systems) evaluation of Illumina mRNA array data identifying biological processes differentially regulated by FAK activity. The threshold was set at a threefold change, and the y axis is the p-value (log scale). Functional annotation groups (x axis) with genes within the leukocyte extravasation signaling shown as the most significant difference between FAK-WT and FAK-KD embryos. (B) Network analyses map within the leukocyte extravasation signaling group. mRNA target changes are represented as nodes, with known direct (solid lines) and indirect (dashed lines) interactions indicated. The asterisks show genes that were identified multiple times in different pathway analyses (shown in A). LDL, low-density lipoprotein; HGF, hepatocyte growth factor; TCR, T cell receptor; WAS, Wiskott–Aldrich syndrome.

Figure S4.  FAK activity is not required for TNF-α–stimulated NF-κB activation. (A) NF-κB EMSA was performed with nuclear extracts isolated from FAK-WT and FAK-KD MEF TNF-α stimulation (10 ng/ml, 30 min), as indicated. The arrow indicates activated NF-κB, and 100-fold unlabeled NF-κB probes were added as a cold competitor. (B) NF-κB EMSA was performed with HUVEC extracts upon TNF-α stimulation (10 ng/ml, 30 min), as indicated. FAK-I pretreatment (1 µM PF271, 1 h) did not block stimulated NF-κB DNA binding upon TNF-α treatment. Excess unbound 32P-labeled probes are present in each lane.
Figure S5. **FAK inhibition blocks TNF-α-induced ERK/MAPK but not NF-κB activation in lung lysates.** (A) Experimental timeline. FAK-I (100 mg/kg, PND-1186) was given 3 h before TNF-α (0.02 mg/kg) or PBS tail vein injection, and lung tissues were collected after 5 min for immunoblotting or after 3 h for evaluation of NF-κB activation. (B) Lung lysates analyzed for FAK Y397 phosphorylation, total FAK, activated ERK (pERK; pT202/pY204), total ERK, and actin. Con, control. (C) Nuclear lysates were isolated from lung tissues, and NF-κB EMSA was performed. Pharmacological FAK inhibition (100 mg/kg, PND-1186) did not change in vivo NF-κB activity upon TNF-α signaling.